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BIOLOGICAL BULLETIN

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August, 1956

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THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-eighth session of the Laboratory.

Except during some of the World War II years, the Laboratory has always operated at capacity in the summer months. However, there have always been more applicants for research space than could be accommodated. The total number of investigators at the Laboratory for the past few years has not been as great as immediately after the war. This has been due in part to the diversion of some laboratories from research to special uses such as radioisotope laboratories, electron-microscopy, and for special equipment, in all, some six laboratories. Preference is given to those investigators who can devote the major share of the summer to their projects and who use the marine biological materials available in the Woods Hole area. However, there is not a rigid adherence to these two factors. The Laboratory is primarily interested in fostering and promoting outstanding biological research, in its broadest terms, and in developing scientists. The allotment of space, however, is becoming more difficult due to an increasing number of applications for research space. For this year an increased number of well qualified investigators cannot be accommodated.

1. Plant Changes and Improvements

Through a grant of $50,000 from the National Science Foundation the extensive repairs of the hurricane damage of 1954 were completed and some modifications were made in the Main Building and Pump House to prevent a recurrence of the flooding of these buildings such as occurred in the 1954 hurricanes. Included were the bricking up of some of the basement windows in the Main Building and in the Pump House as well as other stand-by measures to be used in an emergency. It was necessary to replace and relocate considerable equipment which was irreparably damaged by salt water.

2. Pension Plan

During the past year a study was made of a number of pension plans in force in other organizations with a view to developing a pension plan for the full-time employees of the Laboratory. A plan was developed and became effective September 1, 1955. The employees of the Laboratory were already under Social Security. The Laboratory's pension plan will supplement Social Security benefits. The entire cost of the pension plan will be borne by the Laboratory. For the past 26 years the Laboratory has put aside annually amounts up to 10% of the payroll for pension purposes so there have accumulated funds which are adequate to carry the plan.

3. Grants, Contracts and Contributions

The total income from these sources of support amounted to $192,555.94 in 1955. This represents 39% of the total Laboratory budget and consists of the following accounts:

Amer. Cancer Soc.—026—Function of Nuclei and Nucleic Acids	$ 13,500.00
Amer. Cancer Soc.—R-7F—Fundamental Studies in Radiobiology	6,600.00
A. E. C.-M. B. L.—At 30-1-1343—Program of Research on the Physiology of Marine Organisms Using Radioisotopes	7,674.00
N. I. H.—B6430—Encephalization in Embryonic Development	1,998.00
N. I. H.—SA43PH 423—Investigations of the Microscopic Physiology of Various Forms of Living Marine Life	1,350.00
N. I. H.—B799—Electrical and Mechanical Changes in Muscle	864.00
N. I. H.—RG4359—Biological Research on the Morphology, Ecology, Physiology, Biochemistry and Biophysics of Marine Organisms	40,000.00
National Sci. Found.—G1469—Provision of Funds for Scientific Equipment and Facilities for Biological Research	50,000.00
National Sci. Found.—G1807—Mechano-Chemical Coupling in Muscle	11,500.00
National Sci. Found.—G1395—Osmoregulation of Excretion in Tunicates	1,708.04
O. N. R.—RG4359—Studies in Marine Biology	15,000.00
O. N. R.—09701—Studies on Isolated Nerve Fibers	6,359.34
O. N. R.—09702—Investigation of Environmental Factors Influencing Certain Marine Biological Populations in the Woods Hole Area	4,437.56
American Philosophical Soc.	2,500.00
M. B. L. Associates	2,940.00
Eli Lilly Co.	5,000.00
Rockefeller Found.	20,000.00
Other	1,125.00
	$192,555.94

4. *Instruction*

Dr. Daniel Mazia completed five years as head of the course in Physiology in 1955 and will be succeeded by Dr. Stephen Kuffler. Dr. S. Meryl Rose also completed a five-year term as head of the course in Embryology and will be succeeded by Dr. Mac V. Edds. The success of these courses is attested by the large number of applicants for admission to both of the courses. The Laboratory is to be congratulated in having such excellent leadership in its various courses.

5. *Fellowships and Scholarships*

Under a new plan all of the Lalor Fellows will be selected by a panel set up by the Lalor Foundation. The successful applicants who elect to work at the Marine Biological Laboratory will be permitted to do so. It is hoped that under this new plan the Laboratory will continue to have a fair number of Lalor Fellows. The Laboratory has much to offer these young postdoctoral fellows and would not realize its full educational potentialities without them. Also these fellowships have been of real value to the Laboratory, resulting in the recruitment of highly qualified investigators in subsequent years.

6. Losses by Death

Since the last report the Laboratory has lost through death two eminent scientists who served for many years on the Board of Trustees, Professor George Howard Parker and Professor Warder Clyde Allee. Both of these scientists contributed in an effective way to the prestige and development of the Laboratory.

Respectfully submitted,

PHILIP B. ARMSTRONG,
Director

1. THE STAFF, 1955

PHILIP B. ARMSTRONG, Director, State University of New York, School of Medicine, Syracuse

SENIOR STAFF OF INVESTIGATION

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G. H. PARKER, Professor of Zoology, *Emeritus,* Harvard University

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LIBBIE H. HYMAN, American Museum of Natural History
A. C. REDFIELD, Woods Hole Oceanographic Institution

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JOHN H. LOCHHEAD, Associate Professor of Zoology, University of Vermont
NORMAN A. MEINKOTH, Associate Professor of Zoology, Swarthmore College
GROVER STEPHENS, Assistant Professor of Zoology, University of Minnesota
JOHN M. ANDERSON, Associate Professor of Zoology, Cornell University
MURIEL SANDEEN, Department of Zoology, Duke University
L. M. PASSANO, Department of Zoology, University of Washington, Seattle
MORRIS ROCKSTEIN, Department of Physiology, New York University, Bellevue Medical Center

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DOROTHY M. SKINNER, Tufts College

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NELSON T. SPRATT, JR., Professor of Zoology, University of Minnesota
J. P. TRINKAUS, Assistant Professor of Zoology, Yale University
EDGAR ZWILLING, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistants

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Roger D. Milkman, Harvard University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California, in charge of course
Herman M. Kalckar, National Institutes of Health
Stephen Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins University Medical School
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
George Wald, Professor of Biology, Harvard University
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Columbia University

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Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

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Robert W. Krauss, University of Maryland
Richard C. Starr, Instructor in Botany, University of Indiana

III. Laboratory Assistants

Francis R. Trainor, Vanderbilt University

IV. Collector

Ann Allen, University of Indiana

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II. Instructors

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
Edwin T. Moul, Assistant Professor of Botany, Rutgers University
Charles Jenner, Associate Professor of Zoology, University of North Carolina

III. Assistant

Rudolf Scheltema, George Washington University

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Donald P. Costello, Managing Editor
University of North Carolina, Dept. of Zoology
Chapel Hill, North Carolina
Catherine Henley, Assistant to the Editor

2. Investigators and Students

Independent Investigators, 1955

Abbott, Robinson Shewell, Assistant Professor of Botany, Cornell University
Agnew, L. R. C., Research Fellow, Department of Nutrition, Harvard University
Allen, Robert Day, Instructor in Zoology, University of Michigan
Anderson, John Maxwell, Associate Professor of Zoology, Cornell University
Armstrong, Philip B., Professor of Anatomy, State Univ. of New York, College of Medicine
Arnold, William A., Scientific Investigator, Oak Ridge National Laboratory
Barton, Jay, Assistant Professor of Zoology, Columbia University
Bennett, Miriam F., Instructor in Biology, Sweet Briar College
Berger, Charles A., Chairman, Department of Biology, Fordham University
Blum, Harold E., Physiologist, Princeton University
Boettiger, Edward G., Associate Professor, University of Connecticut
Bold, Harold C., Vanderbilt University
Bridgman, Josephine, Professor of Biology, Agnes Scott College
Brown, Frank A., Jr., Chairman, Dept. of Biological Sciences, Northwestern University
Brummett, Anna Ruth, Instructor in Biology, Carleton College
Bullock, Theodore H., Associate Professor of Zoology, Univ. of California, Los Angeles
Burbanck, W. D., Department of Biology, Emory University
Butler, Elmer G., Professor of Zoology, Princeton University
Chaet, A. B., Instructor in Zoology, University of Maine
Chang, C. Y., Research Associate, State University of Iowa
Chase, Aurin M., Associate Professor of Biology, Princeton University
Cheney, Ralph Holt, Professor of Biology, Brooklyn College
Claff, C. Lloyd, Research Associate in Surgery, Harvard Medical School
Clark, Elliot R., Professor *Emeritus* of Anatomy, Univ. of Pennsylvania School of Medicine
Clayton, Roderick K., Associate Professor of Physics, U. S. Naval P. G. School
Clowes, G. H. A., Research Director *Emeritus*, Eli Lilly and Company
Cole, Kenneth S., Chief, Laboratory of Biophysics, National Institutes of Health
Colwin, Arthur L., Associate Professor and Lecturer, Queens College
Cooperstein, Sherwin J., Assistant Professor of Anatomy, Western Reserve Univ. School of Medicine
Corliss, Clark E., Instructor in Anatomy, University of Tennessee
Costello, Donald P., Kenan Professor of Zoology, Univ. of North Carolina
Crowell, Sears, Assistant Professor of Zoology, Indiana University
Csapo, A., Carnegie Institution of Washington
Dwyer, John D., Director, Dept. of Biology, St. Louis University
Edds, M. V., Jr., Associate Professor of Biology, Brown University
Elliott, Alfred M., Associate Professor of Zoology, University of Michigan
Failla, G., Professor, Columbia University
FitzHugh, Richard, Instructor in Physiological Optics, Johns Hopkins University
Flavin, Martin, Jr., Postdoctoral Fellow, New York University
Freygang, Walter H., Jr., S. A. Surg. (R) U. S. Public Health Service
Gall, Joseph G., Instructor in Zoology, University of Minnesota
Gamow, George, Professor, George Washington University
Gasteiger, Edgar L., Assistant Professor of Physiology, Harvard Medical School
Gilman, Lauren C., Associate Professor of Zoology, University of Miami
Green, James W., Assistant Professor of Physiology, Rutgers University

Green, Maurice, Instructor of Biochemistry, Childrens Hospital, University of Pennsylvania
Gregg, James H., Assistant Professor of Biology, University of Florida
Grobstein, Clifford, Biologist, National Cancer Institute
Grosch, Daniel S., Associate Professor of Genetics, North Carolina State College
Grundfest, Harry, Associate Professor of Neurology, College of Physicians and Surgeons
Guttman, Rita, Assistant Professor of Biology, Brooklyn College
Hagiwara, Susumu, Research Associate, University of California, Los Angeles
Harvey, Ethel Browne, Independent Investigator, Biology Department, Princeton University
Harvey, E. Newton, Professor of Physiology, Princeton, New Jersey
Hay, Elizabeth D., Instructor in Anatomy, Johns Hopkins University School of Medicine
Haywood, Charlotte, Professor of Physiology, Mount Holyoke College
Heilbrunn, L. V., Zoological Laboratory, University of Pennsylvania
Hervey, John P., Electronic Engineer, Rockefeller Institute for Medical Research
Holz, George G., Jr., Assistant Professor of Zoology, Syracuse University
Howard, Robert Stearns, Assistant Professor of Biology, University of Delaware
Hyde, Beal B., Assistant Professor of Plant Sciences, University of Oklahoma
Jacobs, M. H., Professor of General Physiology, Medical School, University of Pennsylvania
Jenner, Charles E., Associate Professor of Zoology, University of North Carolina
Jenkins, George B., *Emeritus* Professor of Anatomy, George Washington University
Kalckar, Herman M., Visiting Scientist, National Institutes of Health
Kavanau, J. Lee, Assistant, Rockefeller Institute for Medical Research
Kempton, Rudolf T., Professor of Zoology, Vassar College
Keosian, John, Professor of Biology, Rutgers University
Kind, C. Albert, Assistant Professor of Zoology, University of Connecticut
King, John W., Professor of Biology, Morgan State College
Kleinholz, L. H., Professor of Biology, Reed College
Klotz, Irving M., Professor of Chemistry, Northwestern University
Krahl, Maurice E., Professor of Physiology, University of Chicago
Kuffler, Stephen W., Associate Professor of Ophthalmology, Johns Hopkins Hospital
Kunkel, Henry G., Rockefeller Institute
Kuntz, Eloise, Assistant Professor, Vassar College
Lansing, Albert I., Professor of Anatomy, Emory University
Lauffer, Max A., Professor and Head of Dept. of Biophysics, University of Pittsburgh
Lazarow, Arnold, Professor and Head of Dept. of Anatomy, University of Minnesota
Levine, Robert P., Assistant Professor, Harvard University
Lewin, Ralph A., National Research Council, Maritime Regional Laboratory, Halifax, N. S.
Levy, Milton, Professor, New York University, Bellevue Medical Center
Lloyd, David P. C., Rockefeller Institute for Medical Research
Lochhead, John H., Professor of Zoology, University of Vermont
Love, Warner E., Associate, Johnson Foundation, Maloney Clinic
Lynch, William F., Professor of Biology, St. Ambrose College
McClement, Patricia, Research Scientist, Columbia University
Marsland, Douglas, Professor of Biology, New York University, Washington Square College
Macchi, Italo A., Assistant Professor of Physiology, Clark University
Mazia, Daniel, Professor of Zoology, University of California
Meinkoth, Norman A., Associate Professor of Biology, Swarthmore College
Menkin, Valy, Head of Experimental Pathology, Temple University School of Medicine
Metz, Charles B., Associate Professor of Zoology, Florida State University
Milkman, Roger, Teaching Fellow, Harvard University
Miller, James A., Professor of Anatomy, Emory University
Moore, John W., Biophysicist, National Institutes of Health
Moul, Edwin T., Associate Professor of Botany, Rutgers University
Mullins, L. J., Associate Professor, Purdue University
Nace, Paul Foley, Associate Professor of Anatomy, New York Medical College
Nelson, Leonard, Assistant Professor of Physiology, University of Nebraska
O'Malley, Benedict B., 160 West 88 Street, New York City 24, New York
Oomura, Yutaka, Research Associate, Neuropsychiatric Institute, University of Illinois
Osterhout, W. J. V., Member *Emeritus*, Rockefeller Institute for Medical Research

Parker, Johnson, Assistant Professor of Botany, University of Idaho
Passano, Leonard M., Instructor, University of Washington
Pierce, Madelene E., Professor of Zoology, Vassar College
Plough, Harold H., Professor of Biology, Amherst College
Prosser, C. Ladd, Professor of Physiology, University of Illinois
Ray, Charles, Jr., Assistant Professor of Biology, Emory University
Ray, David T., Instructor of Zoology, Howard University
Rockstein, Morris, Assistant Professor of Physiology, New York University, Bellevue Medical Center
Rogers, K. T., Assistant Professor of Zoology, Oberlin College
Roys, Chester, Research Associate, Tufts College
Ruben, Laurens Norman, Princeton University
Rugh, Roberts, Associate Professor of Radiology, Columbia University
Sandeen, Muriel I., Assistant Professor of Zoology, Duke University
Schechter, Victor, Associate Professor of Biology, City College of New York
Scott, Allan, Professor of Biology, Colby College
Scott, Sister Florence Marie, Professor of Biology, Seton Hill College
Scott, George T., Professor of Zoology, Oberlin College
Seaman, Gerald R., Associate Professor of Physiology, University of Texas Medical Branch
Shedlovsky, Theodore, Rockefeller Institute for Medical Research
Slifer, Eleanor H., Associate Professor of Zoology, State University of Iowa
Small, Jean E., Graduate Student, Brown University
Solomon, Sidney, Assistant Professor of Physiology, Medical College of Virginia
Spiegel, Melvin, Research Fellow, California Institute of Technology
Speidel, Carl C., Professor and Chairman, Dept. of Anatomy, University of Virginia
Spyropoulos, Constantine, Assistant Scientist, National Institutes of Health
Starr, Richard C., Assistant Professor of Botany, Indiana University
Steele, R. H., Research Fellow, Muscular Dystrophy Association of America
Steinbach, H. B., Professor of Zoology, University of Minnesota
Steinberg, Malcom S., Graduate Student, University of Minnesota
Steinhardt, Jacinto, Director, Operations Evaluation Group, Massachusetts Institute of Technology
Stephens, Grover C., Instructor in Zoology, University of Minnesota
Stephenson, William K., Assistant Professor of Biology, Earlham College
Stokey, Alma G., *Emeritus* Professor of Plant Science, Mount Holyoke College
Stunkard, Horace W., Professor *Emeritus* of Biology, New York University
Szent-Gyorgyi, A. E., Institute for Muscle Research at Marine Biological Laboratory
Takagi, Sadayuki, Research Associate, Neuropsychiatric Institute, University of Illinois
Tasaki, Ichiji, Visiting Scientist, National Institutes of Health
Taylor, Wm. Randolph, Professor of Botany, University of Michigan
Trautwein, Wolfgang, Fellow, Johns Hopkins University Medical School
Trinkaus, J. P., Assistant Professor of Zoology, Osborn Zoological Laboratory, Yale University
Tyler, Albert, Professor of Embryology, California Institute of Technology
Uretz, Robert B., Instructor in Biophysics, University of Chicago
Vincent, Walter S., Instructor in Anatomy, State University of New York College of Medicine
Warner, Robert C., Associate Professor, New York University-Bellevue Medical Center
Webb, Marguerite, Assistant Professor of Physiology, Goucher College
Weiss, Paul, Rockefeller Institute for Medical Research
Weisz, Paul B., Associate Professor of Biology, Brown University
Whiting, P. W., Professor of Zoology, University of Pennsylvania
Wichterman, Ralph, Professor of Biology, Temple University
Wilber, Charles G., Chief, Animal Ecology Branch, Chemical Corps Medical Laboratories
Wilczynski, J., Professor of General Biology and Genetics, Lebanese University, Beirut, Lebanon
Wilbrandt, Walter, Head of Dept. of Pharmacology, University of Berne, Switzerland
Willey, Charles H., Professor of Biology, New York University, Heights
Wilson, Walter L., Assistant Professor of Physiology, University of Vermont College of Medicine

Yntema, Chester L., Professor of Anatomy, State University of New York Upstate Medical Center
Young, R. T., University of Maryland
Zirkle, Raymond E., Professor of Radiobiology, University of Chicago
Zweifach, Benjamin W., Associate Prof. of Biology, New York University, Washington Square College
Zwilling, Edgar, Associate Professor, University of Connecticut

Beginning Investigators, 1955

Aiello, Edward, Assistant in Zoology, Columbia University
Bradford, William Dalton, Medical Student, Western Reserve University School of Medicine
Drake, John W., Graduate Assistant, California Institute of Technology
Geiger, H. Jack, Student, Western Reserve University
Kaye, Alvin M., Assistant Instructor, University of Pennsylvania
Laris, Philip C., Graduate Student, Princeton University
Lavoie, Marcel E., Syracuse University
McKinnell, Robert Gilmore, University of Minnesota
Morrill, John B., Florida State University

Research Assistants, 1955

Aoto, Tomoji, Research Assistant, State University of Iowa
Adams, Terry, Massachusetts Horticultural Society
Allen, M. Ann, Indiana University
Allerand, C., Albany Medical College
Balabanis, Rebecca, South Milwaukee, Wisconsin
Basch, Paul Frederick, University of Michigan
Birsky, Bill, Indiana University
Brown, Robert A., Northwestern University
Dowling, John Alan, Harvard College
Ellis, Gordon W., University of California
Erickson, Joan, Radcliffe College
Friz, Carl T., University of Minnesota
Kahn, Kenneth, University of Pennsylvania
LaChance, Leo E., North Carolina State College
Lefkowitz, Lewis B., Southwestern Medical School
Nathanson, Donald L., Amherst College
Oberlander, Marcia I., State University of New York College of Medicine at Syracuse
Rafferty, Keen A., Jr., University of Illinois
Regehr, Hulda, University of Minnesota
Shelburne, James Christie, Emory University
Skinner, Dorothy M., Radcliffe College
Watt, Donald, Columbia University College of Physicians and Surgeons
Wilt, Fred, Indiana University
Zimmerman, Arthur M., New York University, Washington Square College

Library Readers, 1955

Beck, Lyle V., Associate Professor of Physiology, University of Pittsburgh School of Medicine
Beutner, Reinhard H., Des Moines Still College of Osteopathy
Bodansky, Oscar, Attending Clinical Biochemist, Sloan-Kettering Institute
Crouse, Helen V., Associate Professor, Goucher College
Dean, Helen Wendler, Cambridge, Mass.
DuBois, Eugene, *Emeritus* Professor of Physiology, Cornell University Medical College
Eichel, Herbert J., Research Associate in Biological Chemistry, Hahnemann Medical College
Freund, Jules, Public Health Research Institute of the City of New York
Gabriel, Mordecai, Assistant Professor, Brooklyn College

Gaffron, Hans, Professor of Biochemistry, University of Chicago
Ginsberg, Harold S., Associate Professor of Preventive Medicine, Western Reserve University School of Medicine
Glass, H. Bentley, Professor of Biology, Johns Hopkins University
Goldthwait, David, Associate Member, Dept. of Biochemistry, Western Reserve University
Grant, Philip, Research Associate, Institute for Cancer Research
Greif, Roger L., Associate Professor of Physiology, Cornell University Medical College
Gudernatsch, Frederick, Cornell University Medical College
Johansson, Arne, Foreign Operations Administration, University of Colorado
Johnson, Thomas N., Assistant Professor of Anatomy, George Washington University Medical School
Jones, Sarah R., Instructor in Zoology, Connecticut College
Kabat, Elvin A., Professor of Microbiology, College of Physicians and Surgeons
Karush, Fred, Associate Professor of Immunology, University of Pennsylvania School of Medicine
Katz, Louis Nelson, Professorial Lecturer in Physiology, University of Chicago
Kerschner, Jean, Assistant Professor, Western Maryland College
Kindred, James E., Professor of Anatomy, University of Virginia School of Medicine
Kinersly, Thorn, Research Fellow, Yale University School of Medicine
Kleinfeld, Ruth G., Postdoctoral Fellow, National Cancer Institute, Ohio State University
Kolin, Alexander, Associate Professor of Physics, University of Chicago
Levine, Rachmiel, Chairman, Dept. of Medicine, Michael Reese Hospital
Lippman, Heinz I., Assistant Clinical Professor, Albert Einstein College of Medicine
Loewi, Otto, Research Professor of Pharmacology, N. Y. U., College of Medicine
Love, Lois H., Research Associate, National Research Council
Loewenfeld, Irene E., Research Technician, Columbia University
Lowenstein, Otto, Research Associate, Columbia University
McDonald, Sister Elizabeth Seton, College of Mount St. Joseph on the Ohio
Milstein, Seymour W., Research Associate, Hahnemann Medical College
Nachmansohn, David, Professor of Biochemistry, Columbia University
Pequegnat, Willis, Professor of Zoology, Pomona College
Pick, Joseph, Associate Professor of Anatomy, N. Y. U. Bellevue Medical Center
Ravin, Arnold W., Assistant Professor of Biology, University of Rochester
Robert, Nan L., Instructor in Biological Sciences, Hunter College
Root, Walter S., Professor of Physiology, College of Physicians and Surgeons
Rothstein, Fred, Hahnemann Medical College
Schlesinger, R. Walter, Associate Member, The Public Health Research Institute of New York
Schneyer, Leon H., Associate Professor of Clinical Dentistry, College of Alabama
Schwabe, Louise A., Science Teacher, Kenmore Senior High School
Smelser, George K., Professor of Anatomy, Columbia University
Sulkin, S. Edward, Professor of Microbiology, Southwestern Medical School
Tauber, Hans-Lukas, Assistant Professor, New York University College of Medicine
Wainio, Walter W., Associate Professor of Biochemistry, Rutgers University
Waksman, Bryon H., Associate in Bacteriology, Harvard University
Weidman, S., State University of New York College of Medicine at Brooklyn
Wheeler, George E., Instructor in Biology, Brooklyn College

Students, 1955

BOTANY

Ahmadjian, Vernon, Clark University
Arce, Gina, Vanderbilt University
Cashman, Marjean L., University of Maryland
Courtenay, Walter Rowe, Jr., Vanderbilt University
Cox, Samuel F., Vanderbilt University
Cross, Caroline B., Acadia University

Eiger, Joan V., City College of New York
Freudenthal, Hugo D., Columbia University, College of Pharmacy
Galloway, Raymond A., University of Maryland
Gates, John, Cornell University
Green, Paul B., Princeton University
Hilferty, Frank, State Teachers College
Hoffer, Joseph L., Fordham University
Lamb, Ivan Mackenzie, Harvard University
Overstreet, Rose Alice, Indiana University
Pokorny, Frank J., St. John's University
Rader, Philip Scott, Middle Tennessee State College
Scheltema, Rudolf S., Harvard University
Wilson, Vannie William, Morgan State College

EMBRYOLOGY

Bagnara, Joseph T., State University of Iowa
Beard, Robert Gordon, Indiana University
Borodach, Gerold N., Brown University
Bourke, Robert Samuel, Harvard University
Carey, Francis Gerald, Harvard University
Davis, Rowland Hallowell, Harvard University
de la Paz, Justo, Cornell University
de Terra, Noel, Barnard College
Goldstein, Joel B., Haverford College
Greenless, Janet Lucile, University of Wisconsin
Harris, Patricia J., Yale University
Hickson, Elizabeth, Brown University
Knepton, James C., Jr., Duke University
Laufer, Wila P., Tufts University
Lyser, Katherine May, Oberlin College
McArdle, Eugene William, Marquette University
Matheson, Gail E., Wheaton College
Mendelsohn, Everett, Harvard University
Nathanson, Donald Lawrence, Amherst College
Neu, Harold C., Creighton University
Orelup, Alethea Ann, University of Illinois
Pierce, Peter G., Colby College
Rafferty, Nancy S., University of Illinois
Sage, Janet Kathleen, DePauw University
Schultes, Sandra Jean, Goucher College
Shoger, Ross L., University of Minnesota
Skinner, Dorothy M., Radcliffe College
Spencer, Charles David, Wesleyan University
Tull, Dade Louise, Vassar College
Young, Robert Rice, Yale University

PHYSIOLOGY

Baker, K. France, Columbia University
Cazorla, F. Alberto, Instituto NCNAL de Enferme-Dades Neoplasicas, Lima, Peru
Cordeau, Jean Pierre, Université de Montreal
Crocker, Charity S., Instituto de Biofisica, Rio de Janeiro, Brazil
Curry, George Montgomery, Harvard University

Ekberg, Donald Roy, University of Illinois
Fernandes, Jose Ferreira, Medical Faculty, Sao Paulo, Brazil
Fleisher, Joseph H., Chemical Corps, Medical Laboratories
Fleming, William Wright, Harvard University
Goe, Don Richard, University of Southern California
Goldsmith, Timothy H., Harvard University
Guccione, Ignatius, New York University
Herranen, Ailene M., University of Wisconsin
Hurwitz, Charles, V. A. Hospital, Albany, New York
Kepchar, John Howard, University of North Carolina
Lefkowitz, Lewis B., Jr., Southwestern Medical School
Levine, Laurence, Wayne University
MacHattie, Lorne Allister, University of Buffalo
Mauzerall, David C., Rockefeller Institute for Medical Research
Mendelsohn, Mary L., Radcliffe College
Messineo, Luigi, Institute of Zoology of Palermo, Italy
Neuhaus, Francis C., Duke University
Rhodes, William C., Johns Hopkins University
Roberts, Jane C., University of California, Los Angeles
Rogers, Palmer, Johns Hopkins University
Small, Arlene May, Mount Holyoke College
Stevens, Carl Mantle, State College of Washington
Urban, Theodore J., Creighton University
Wahba, Albert J., Cornell University
Wohlhieter, John Andrew, University of Pittsburgh

ZOOLOGY

Abbott, Joan, Washington University
Adam, Betty Rose, De Paul University
Babcock, Richard G., University of Michigan
Bates, Griffin Miller, Hamilton College
Beard, Robert G., Indiana University
Bishop, Jane Ellen, Oberlin College
Brown, Edward R., University of Cincinnati
Bruening, Betty L., Goucher College
Butz, Andrew, Fordham University
Cali, Carmen T., Fordham University
Camougis, George, Harvard University
Carey, Francis Gerald, Harvard University
Christensen, Albert Kent, Harvard University
Church, Charles Henry, Jr., Wesleyan University
Cranston, Margaret B., Mount Holyoke College
Davis, Peter Wright, Bowdoin College
Dickinson, Winifred, Pennsylvania College for Women
Drury, George L., S. J., Boston College
Dungan, Shirley R., DePauw University
Eakin, Edwin Lloyd, Kenyon College
Fabiny, Robert John, Marquette University
Fassuliotis, George, New York University
Feeley, Edward J., Fordham University
Finlay, Peter S., Syracuse University
Gates, John Otis, Cornell University
Golden, Harold J. J., Saint Louis University
Gross, Charles, Harvard College

Hall, William H., University of Virginia
Hofstetter, Sister Adrian Marie, Notre Dame University
Horrell, Harry Cunningham, University of Chicago
Josephson, Robert Karl, Tufts University
Kinyon, Nancy, Northwestern University
Lisa, Joseph D., Fordham University
MacHattie, Lorne A., University of Buffalo
Markham, Alice Elinor, Mount Holyoke College
Mauriello, George E., New York University
Menges, Elizabeth V., Smith College
Orr, Antoinette M., Marlboro College
Ozburn, George W., Ontario College
Panuska, Joseph Allen, St. Louis University
Parsons, John A., Pennsylvania State University
Pascale, Jane Fay, University of Chicago
Peacock, Ronnie, Earlham College
Picciano, Antoinette A., Northwestern University
Ross, Donald J., Fordham University
Rousall, Paul G., S. J., Fordham University
Salyerds, Anne Martha, Agnes Scott College
Scott, Diana F., Swarthmore College
Smith, Sally, Vassar College
Spano, Rev. Anthony A., Fordham University
Swader, Laura Lynn, Drew University
Taylor, Peter B., Cornell University
Truong, Rev. Hoang, Northwestern University
Whitin, Navamonie, Wellesley College
Wischnitzer, Saul, University of Notre Dame
Woods, James E., DePaul University

ECOLOGY

Bing, Peter S., Los Angeles 24, California
Carey, Andrew G., Princeton University
Cohen, Matanah, University of Colorado
Davis, Roger E., University of Wisconsin
Dial, Norman A., University of Illinois
Felitti, Vincent Justus, Dartmouth College
Freudenthal, Anita R., New York University
Freudenthal, Hugo D., Columbia University
Haws, Clayton, Drew University
McLaughlin, John J., Haskins Laboratories, New York University
Morrill, John B., Jr., Florida State University
Nagle, Mary Elizabeth, Clark University
Wright, Theodore, Yale University

3. Lalor Fellows, 1955

Clayton, Roderick K., U. S. Naval P. G. School
Gest, H., Western Reserve University
Green, Maurice, Childrens Hospital, University of Pennsylvania
Lewin, Ralph, National Research Council, Maritime Regional Laboratory, Halifax, N. S.
Macchi, Italo, Clark University
Roth, Jay, Hahnemann Medical College
Wilbrandt, W., University of Berne, Switzerland

4. Tabular View of Attendance, 1951–1955

	1951	*1952*	*1953*	*1954*	*1955*
INVESTIGATORS—TOTAL	303	306	310	298	250
Independent	186	172	176	180	162
Under Instruction	28	38	37	20	9
Library Readers	37	49	46	52	54
Research Assistants	52	47	51	46	25
STUDENTS—TOTAL	124	123	136	134	148
Zoology	55	55	55	56	56
Embryology	27	23	30	29	30
Physiology	29	27	31	28	30
Botany	13	11	11	12	19
Ecology		7	9	9	13
TOTAL ATTENDANCE	427	429	446	432	398
Less persons represented as both students and investigators		2		5	
	427	427	446	427	398
INSTITUTIONS REPRESENTED—TOTAL	158	149	155	136	129
By Investigators	115	92	90	104	95
By Students	43	57	65	32	34
SCHOOLS AND ACADEMIES REPRESENTED					
By Investigators	1	1		2	3
By Students		3	1	1	2
FOREIGN INSTITUTIONS REPRESENTED					
By Investigators	8	7	15	11	8
By Students	3	2	6	13	6

5. Cooperating and Subscribing Institutions, 1955

Cooperating Institutions

Amherst College
American Cancer Society
American Philosophical Society
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph on the Ohio
Columbia University
Columbia University, College of Physicians and Surgeons
Cornell University
Cornell University Medical School
Duke University
Elmira College
Emory University
Florida State University
Fordham University
George Washington University Medical School
Grass Foundation
Hahnemann Medical College
Harvard University
Harvard University Medical School
Institute for Cancer Research
Institute for Muscle Research
Johns Hopkins University
Johns Hopkins University Medical School
Lalor Foundation
Eli Lilly and Company
Morgan State College
Mount Holyoke College
National Institutes of Health
National Science Foundation
New York University, College of Medicine
New York University—Heights
New York University—Washington Square College
North Carolina State College
Northwestern University
Oberlin College
Office of Naval Research
Princeton University
Public Health Institute of New York
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Saint Louis University
Sloan-Kettering Institute

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Southwestern Medical College
State University of Iowa
State University of New York, College of Medicine, at Syracuse
Syracuse University
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Delaware
University of Illinois
University of Maryland School of Medicine
University of Michigan
University of Minnesota
University of Nebraska
University of North Carolina
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Virginia, School of Medicine
University of Wisconsin
Vassar College
Washington University
Washington and Jefferson College
Wesllesley College
Wesleyan University
Western Reserve University
Yale University

Subscribing Institutions

Acadia University
Boston College
Ethicon Corporation
Goucher College
Guggenheim Foundation
Hamilton College
Indiana University
Marquette University
Pennsylvania College for Women
Purdue University
Radcliffe College
Saint Ambrose College
State University of New York at Brooklyn
University Center of Georgia
University of Alabama School of Dentistry
University of Illinois, College of Medicine
University of Maine
University of Oklahoma
University of Texas Medical School
Yale University School of Medicine

6. EVENING LECTURES, 1955

June 24
PAUL WEISS .... "Some thoughts and experiments on morphogenesis"

July 1
GEORGE WALD .... "The origin of life—some special problems"

July 8
F. SJOSTRAND .... "Ultrastructural organization of retinal receptor cells"

July 15
BRADLEY M. PATTEN .... "Micromoving picture studies of the first heart beats in the beginning of the embryonic circulation"

July 22
F. O. SCHMITT .... "Chemical and structural studies of nerve fibers"

July 29
WALTER WILBRANDT .... "Carrier transport systems and their kinetics"

August 5
F. A. BROWN, JR. .... "The rhythmic nature of life"

August 12
ARNOLD LAZAROW .... "Diabetic toadfish; their use in studies on the etiology of diabetes mellitus"

August 19
KENNETH S. COLE .... "Squid axon excitation"

August 26
GEORGE KIDDER .... "Metabolic studies on animal microorganisms"

7. TUESDAY EVENING SEMINARS, 1955

July 5	
R. P. LEVINE	"Cations in chromosome structure: their relation to the mechanism of crossing over"
DANIEL MAZIA and WALTER S. PLAUT	"Distribution of parental material in chromosome reproduction"
MAX A. LAUFFER and HERMAN CEMBER	"The effects of ionizing radiations on cockroach embryos"
July 12	
J. P. TRINKAUS and PEGGY W. GROVES	"Differentiation of mixed aggregates of dissociated tissue cells"
R. E. ZIRKLE, W. BLOOM and R. B. URETZ	"Chromosome movements and cell division after spindle destruction by irradiation of cytoplasm"
R. B. URETZ, W. BLOOM and R. E. ZIRKLE	"Changes in refractive index of irradiated chromosome segments"
July 19	
GERALD R. SEAMAN	"Purification and properties of an enzyme system which reversibly cleaves succinate to join two molecules of acetyl coenzyme A"
JAY S. ROTH	"Ribonuclease inhibition"
ELIZABETH ANDERSON	"Metabolism of uridinediphosphoglycosyl compounds"
ALEXANDER KOLIN	"Some recent experiments on electrokinetic separation of proteins and of microorganisms"
July 26	
R. K. CLAYTON	"Tactic responses of purple bacteria"
WARNER E. LOVE	"The molecular weight of hemerythrin of *Phascolosoma gouldi* by x-ray diffraction"
BARBARA E. WRIGHT	"Pteridine coenzymes in one carbon metabolism"
P. F. SCHOLANDER	"Secretion of inert gases and oxygen by the swim-bladder of fishes"
August 2	
CARL C. SPEIDEL	"Motion pictures of cellular changes in tadpoles following x-ray irradiation"
ROBERT ALLEN	"Protoplasmic streaming and amoeboid movement"
S. G. A. ALIVISATOS	"Enzymic synthesis of new dinucleotides by a novel method of biosynthesis"
PAUL S. GALTSOFF	"Structure and function of the ligament of Pelecypoda"
August 9	
E. G. BUTLER and H. F. BLUM	"Effects of ultraviolet on regenerative activity in urodeles"
S. HAGIWARA and T. H. BULLOCK	"Study of intracellular potentials in pacemaker and integrative neurons of the lobster cardiac ganglion"
T. H. BULLOCK and S. HAGIWARA	"Further study of the giant synapse in the stellate ganglion of squid"
CHARLES G. WILBER	"Electrocardiogram of the alligator"

August 16	
Anna R. Whiting and William E. Murphy	"Differences in response of x-rayed eggs and spermatozoa of Habrobracon to anoxia"
Ralph A. Lewin	"Paralysis in double-mutants of Chlamydomonas"
H. H. Plough and Margaret Roberts	"High frequency of transduction of genes by bacteriophage in Salmonella"
Joyce C. Lewin	"Physiological races of the diatom, *Navicula pelliculosa*"
August 23	
Philip Grant	"Some observations on the incorporation of glycine C-14 into amphibian embryos"
Maurice Green	"Fucose metabolism in *Escherichia coli*"
James A. Miller, Jr.	"The potentiation by narcosis of the beneficial effects of hypothermia in asphyxia of the neonatal guinea pig"
W. Wilbrandt and P. Luescher	"Action of a thrombocyte protein on capillary permeability"

8. Members of the Corporation. 1955

1. Life Members of the Corporation

Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts
Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania
Carver, Prof. Gail L., Mercer University, Macon, Georgia
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri
Dederer, Dr. Pauline H., Connecticut College, New London, Connecticut
Dungay, Dr. Neil S., Carleton College, Northfield, Minnesota
Goldfarb, Dr. A. J., College of the City of New York, New York City
Jackson, Mr. Charles C., 24 Congress Street, Boston, Massachusetts
Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts
King, Mr. Charles A.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland
Lowther, Dr. Florence deL., Barnard College, New York City, New York
MacNaught, Mr. Frank M., Woods Hole, Massachusetts
Malone, Prof. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Missouri
Moore, Dr. J. Percy, University of Pennsylvania, Philadelphia, Pennsylvania
Noyes, Miss Eva J.,
Payne, Dr. Fernandus, Indiana University, Bloomington, Indiana
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania
Riggs, Mr. Lawrason, 120 Broadway, New York City, New York
Scott, Dr. Ernest L., Columbia University, New York City, New York
Sears, Dr. Henry F., 86 Beacon Street, Boston, Massachusetts
Shedd, Mr. E. A.
Waite, Prof. F. G., 144 Locust Street, Dover, New Hampshire

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V. REPORT OF THE LIBRARIAN

In 1955, the number of currently-received journals totalled 1554 (51 new). Of these titles, there were 467 (5 new) Marine Biological Laboratory subscriptions; 604 (10 new) exchanges and 179 (13 new) gifts; 74 (2 new) were Woods Hole Oceanographic Institution subscriptions; 183 (12 new) were exchanges and 47 (9 new) were gifts.

The Laboratory purchased 56 books, received 84 complimentary copies (10 from authors and 74 from publishers) and 60 miscellaneous presentations. The Institution purchased 28 titles. The total number of new books accessioned amounted to 228.

By purchase, the Laboratory completed 6 journal sets and partially completed 13 sets. The Institution completed two sets and partially completed two sets. Volumes and numbers received by gift and by exchange completed 13 sets and partially completed 10 sets.

There were 3891 reprints added to the collection, of which 2317 were of current issue.

The Library sent out on inter-library loan 190 volumes and borrowed 57 for the convenience of the investigators. A sum of $246.43 was realized from the sale of duplicate material.

At the end of the year, Dr. E. Newton Harvey presented to the Laboratory his large and valuable collection of 12,000 reprints. Of these, 2500 were added to the shelves, many of which filled in sets, making them of far greater value than heretofore. This gift is greatly appreciated and grateful acknowledgment is herewith conveyed to the donor. The duplicates of the reprints already in the Library's possession will be sent to another library of Dr. Harvey's choosing.

Other gifts of reprints and books were received from Drs. Wm. R. Amberson, Roberts Rugh, Kurt G. Stern and Ralph A. Lewin. Without these generous contributions, the Library would be minus many worthwhile acquisitions.

At the end of the year, the Library contained 65,463 bound volumes and 196,089 reprints.

During the years 1914–1923, Dr. R. P. Bigelow served as Librarian. In the years following, he did not lose interest in the growth and development of the Library and throughout the past ten years, he made many generous and valuable contributions. In keeping with his thoughtfulness, Mrs. Bigelow presented four fine portraits which had hung in her husband's study, namely, those of Brooks, Darwin, Huxley and Lamarck. Through Dr. Bigelow's death, the Library has lost a great friend.

Respectfully submitted,

DEBORAH L. HARLOW,
Librarian

VI. REPORT OF THE TREASURER

Marine Biological Laboratory

Balance Sheet

December 31, 1955

Investments

Investments held by Trustee:	
Securities, at cost (approximate market quotation $1,500,773)	$ 979,202
Cash	1,441
	980,643
Investments of other endowment and unrestricted funds:	
Pooled Investments, at cost (approximate market quotation $230,368)	213,784
Other investments (note B)	53,076
Cash	9.155
	276,015

Plant Assets

Land, buildings, library and equipment (note A)	2,406,077
Less allowance for depreciation (note A)	955,753
	1,450,324

Current Assets

Cash	36,857
Accounts receivable ($3,596 from U. S. Government)	34,754
Inventories of specimens and Bulletins	61,158
Prepaid insurance and other	8,432
	$2,848,183

Notes:

A—The Laboratory has since January 1, 1916, provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

B—The Laboratory has guaranteed a note of approximately $4,000 of the M. B. L. Club and has pledged as security, therefore, bonds with an original cost of $7,900, included in other investments.

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1955

Endowment Funds

Endowment funds given in trust for the benefit of the Marine Biological Laboratory		$ 980,643
Endowment funds for awards and scholarships:		
Principal	31,738	
Unexpended income	1,377	33,115
Unrestricted funds functioning as endowment		206,378
Retirement fund		35,408
Pooled investments—accumulated gain		1,114
		276,015

Plant Liability and Funds

Mortgage payable on demand, 5%		5,000
Funds expended for plant, less retirements	2,401,077	
Less allowance for depreciation charged thereto	955,753	1,445,324
		1,450,324

Current Liabilities and Funds

Accounts payable	5,767
Unexpended balances of gifts for designated purposes	8,229
Advance payments on research contracts	7,646
Current fund	119,559
	$2,848,183

Marine Biological Laboratory

Statement of Operating Expenditures and Income

Year Ended December 31, 1955

Operating Expenditures

Direct expenditures of departments:	
Research and accessory services	$145,306
Instruction	23,967
Library, including book purchases	30,389
Biological Bulletin	12,684
	212,346
Administration and general	46,988
Plant operation and maintenance	68,974
Hurricane emergency repairs	22,059
Dormitories and dining services	128,460
Equipment purchased from current funds	1,712
	480,539
Less depreciation included in plant operation and auxiliary activities above but charged to plant funds	36,429
	444,110

Income

Direct income of departments:	
Research fees	40,194
Accessory services (including sales of biological specimens, $67,436)	93,230
Instruction fees	17,930
Library fees and income	6,537
Biological Bulletin, subscriptions and sales	15,920
	173,811
Allowance for indirect costs on research contracts	15,747
Dormitories and dining services income	100,768
	290,326
Investment income	73,581
Gifts for current use	121,053
Sundry income	394
Total current income	485,354
Excess of income	$ 41,244

Direct costs on research contracts and reimbursement therefor, $41,311, are not included in operating expenditures or income.

MARINE BIOLOGICAL LABORATORY

STATEMENT OF CURRENT FUND

Year Ended December 31, 1955

Balance January 1, 1955	$ 84,586
Less:	
Amount transferred to unrestricted funds functioning as endowment as of July 15, 1955	6,843
	77,743
Add:	
Provision for uncompleted repairs included in operating expenditures	572
Excess of income over operating expenditures, 1955	41,244
Balance December 31, 1955	$119,559

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS

December 31, 1955

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1955
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$184,206	22.4	$ 178,437	14.4	$ 4,337
Other bonds	285,429	34.8	288,716	23.3	9,079
	469,635	57.2	467,153	37.7	13,416
Preferred stocks	85,788	10.4	80,688	6.5	3,370
Common stocks	265,663	32.4	691,929	55.8	25,148
	821,086	100.0	1,239,770	100.0	41,934
General Education Board endowment fund:					
U. S. Government bonds	48,139	30.4	46,633	17.9	1,113
Other bonds	30,637	19.4	30,425	11.6	997
	78,776	49.8	77,058	29.5	2,110
Preferred stocks	27,281	17.3	26,637	10.2	1,130
Common stocks	52,059	32.9	157,308	60.3	5,350
	158,116	100.0	261,003	100.0	8,590
Total securities held by Trustee	$979.202		$1,500,773		$50,524

MARINE BIOLOGICAL LABORATORY

SUMMARY OF INVESTMENTS—*Continued*

December 31, 1955

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1955
Investments of other endowment and unrestricted funds:					
Pooled investments:					
U. S. Government bonds......	$ 40,349	18.9	$ 38,231	16.6	$ 952
Other bonds................	82,428	38.5	81,455	35.4	2,007
	122,777	57.4	119,686	52.0	2,959
Common stocks.............	91,007	42.6	110,682	48.0	4,382
	213,784	100.0	230,368	100.0	7,341
Other investments:					
Bonds......................	7,920				330
Investment in General Biological Supply House, Inc.	12,700				17,780
Real estate and mortgage.....	32,456				
	53,076				18,110
Total investments of other endowment and unrestricted funds..................	$266,860				$25,451
Total investment income received....................					75,975
Custodian's fees charged thereto....................					(254)
Investment income....................					$75,721

STUDIES ON CROSS–FERTILIZATION AND SELF–FERTILIZATION IN LYMNAEA STAGNALIS APPRESSA SAY[1]

GERTRUDE L. CAIN[2]

Department of Zoology, University of Wisconsin, Madison, Wisconsin

In 1817 Oken obtained fertile eggs from *Lymnaea auricularis* which were reared in isolation during their entire reproductive period. Baudelot (1863) reported both self-fertilization and cross-fertilization in *Lymnaea*. Pelseneer (1920) saw only one polar body extruded from the eggs of *Lymnaea* (three species), and concluded that reproduction in isolated snails was parthenogenetic. However, Colton, (1918) in *L. columella* and Crabb (1927a) in *L. stagnalis* observed two polar bodies and on the basis of their observations concluded that parthenogenesis did not occur. Colton further reported that, although self-fertilization did occur, cross-fertilization was the rule; Crabb reported that cross-fertilization was mechanically impossible (1927b). Seshaiya (1927) concluded from a study of breeding habits of *L. luteola* that both cross- and self-fertilization occurred in this species.

Lang in 1900 claimed that self-fertilization could occur without self-copulation, while Künkel (1908) believed that self-copulation was indispensable to self-fertilization, basing his opinion in part on the observation of self-copulation in *L. auricularis* by Von Baer in 1835. Colton and Pennypacker (1934) reported that self-fertilization in *L. columella* for 93 generations did not decrease the viability of the strain. Boettger (1944), in his survey of the Basommatophora, concluded that self- and cross-fertilization were both common in this order. DeWitt (1954) found the percentage of hatching less in self-fertilized eggs of *Physa gyrina* than in cross-fertilized eggs.

The first genetic proof that both self- and cross-fertilization occur in snails was supplied by Diver, Boycott and Garstang (1925) in a study of the inheritance of inverse symmetry in *L. peregra*. Further proof was obtained in the study of the inheritance of albinism in this snail (Boycott and Diver, 1927). Ikeda and Mura (1934), using shell color as a genetic marker, demonstrated that both self- and cross-fertilization occurred in the land snail, *Bradybaena similaris*.

Bretschneider (1948a, 1948b) investigated the mechanism of insemination and oviposition in *L. stagnalis*. He reported that he had seen sperm balls leaving the seminal vesicle and being swept up the female tract to the hermaphroditic duct, where he assumed fertilization occurred. As additional evidence he reported seeing a complete spermatozoon inside the cytoplasm of an egg still in the duct.

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[1] Revised from a dissertation presented to the graduate School of the University of Wisconsin in partial fulfillment of requirements for the degree of Doctor of Philosophy. The author wishes to express gratitude to Professor L. E. Noland for his kindness and assistance during the course of this study. She is also grateful to the General Education Board and the National Medical Foundation Inc. (funds contributed by the National Foundation of Infantile Paralysis) for financial assistance during the time this study was being pursued.

[2] Present address: West Virginia State College; Institute, West Virginia.

Holm (1946), in micro-anatomical studies on the reproductive tract of *L. stagnalis,* found a "fertilization pocket" homologous to that found by Meisenheimer (1912) in *Helix pomatia,* but saw no eggs or spermatozoa in it. Perrot (1940) reported a similar structure in *Limax maximus.* Abdel-Malek (1954a, 1954b) saw ova in the corresponding pockets in *Helisoma trivolvis* and *Biomphalaria biossyi.*

The present study was undertaken to determine: (1) the mode of inheritance of albinism in *Lymnaea stagnalis;* (2) the relative frequency of self-fertilization as compared with cross-fertilization in this species; (3) the survival time of spermatozoa after transfer from one snail to another; and (4) the possible location of fertilization of the eggs in this snail.

Materials and Methods

The snails used in this study were obtained from strains that had been maintained in laboratory culture at the University of Wisconsin for over ten years. Culture methods were those of Noland and Carriker (1946).

Mode of Inheritance of Albinism

Although albinism has been found to be inherited as a simple Mendelian recessive in several other gastropods (Boycott and Diver, 1927, in *L. peregra;* Ikeda, 1937, in *Philomycus bilineatus*), it was necessary to verify this for *L. stagnalis* before albinism could be used as a genetic marker in this study.

Accordingly two snails, one from the albino culture and one from the pigmented culture, were isolated until each had deposited at least one egg mass. The offspring from the eggs of the pigmented snail were all pigmented and those from the albino snail were all albinos. The two parent snails were then paired for 42 days. During this time one copulation was observed with the pigmented snail serving as the male. Presumably other such copulations occurred when the snails were not under observation.

After 42 days the two snails were separated. The albino was kept in isolation culture, and its egg masses were collected. The offspring resulting from these egg masses were examined under a binocular microscope six days after hatching. By this time pigment had developed along the mantle collar. Any young not showing pigment were re-examined after another five or six days. Of 885 offspring grown from 28 egg masses laid by the albino snail, 43 were albinos, resulting presumably either from self-fertilization or from previous copulations with other albinos in the original stock culture. The 842 pigmented offspring of the albino parent were clearly the result of fertilization of the eggs of the albino by spermatozoa from its pigmented mate.

Six of these pigmented heterozygotes were selected and isolated before sexual maturity. Each was maintained in solitary culture to insure that only self-fertilization would occur. This self-fertilization is obviously the equivalent of crossing two F_1 heterozygotes. Five or more egg masses were saved from each snail, and the progeny therefrom were grown to the age of "pigment-testing." Of a total of 4909 eggs (49 egg masses) from the six heterozygous snails, 64.7% hatched, and of those that hatched 92.9% survived to be examined for the presence of pigment. Of 2949 thus surviving, 2193 were pigmented and 765 were albinos. On a 3:1 basis the expected ratio would have been 2212:737. The agreement is close and, on the basis of chi square tests, the difference between the expected and the observed ratio was not significant. It may therefore be concluded with confidence that albinism in *L. stagnalis* is inherited as a simple Mendelian recessive, as in other gastropods.

Prevalence of Self-fertilization

Eighteen albino snails (15 with pedigreed albino parentage and 3 from exclusively albino stock cultures) were paired, each with a homozygous pigmented snail taken from the stock culture which for ten years had shown no albinos. Each of these pairs was kept in a separate dish for varying lengths of time (from 20 to 187 days, depending on the pair). The albino partners were thereafter maintained in isolation culture. The eggs produced by these albinos were saved until the hatching snails reached the "pigment-testing" age to determine the relative numbers of pigmented and albino progeny. It was assumed that the albino progeny resulted from self-fertilization and the pigmented offspring from cross-fertilization. There was a slight possibility that the three snails, taken as adults from albino cultures, might have cross-copulated with other albinos before isolation. Two of these three snails showed 100% pigmented offspring in their first egg mass. The third was never seen to copulate with its pigmented partner, and produced only albino offspring throughout its life. Since all other snails were young (less than 130 days) and since no copulations had been observed in the cultures from which they were taken, the possibility that they had already cross-copulated with other albinos is extremely small.

Of the 18 albino snails paired with pigmented mates, 15 of them after separation produced mainly pigmented offspring during the first month, while three gave only albino progeny. From this it is clear that, when albino and pigmented snails are paired, not only does cross-fertilization occur, but, contrary to the opinion of Crabb (1927b), it is the predominating process.

Ten of the 15 albino snails that produced pigmented offspring after separation from their pigmented mate gave 100% pigmented young in at least one of their egg masses. In five of these it was the first egg mass laid after isolation that gave only pigmented progeny. One showed only albinos in its first egg mass, but by its third egg mass was producing 100% pigmented young. Of the ten snails that gave 100% pigmented offspring in at least one egg mass, four were producing albinos exclusively by the end of their lives. Three others, however, were still producing 100% pigmented progeny in the last egg mass laid before they died. Noland and Carriker (1946) have shown that snails maintained in solitary culture their entire lives frequently will produce more fertile eggs than snails allowed to cross-copulate. It is therefore unlikely that the continued production of pigmented offspring by the three snails mentioned above could have been due to any lack of fertilizing ability on the part of the animal's own sperm.

While cytological tests were not made to eliminate the possibility of parthenogenesis in the case of isolated snails, this seems unlikely because of the almost exact 3:1 ratio obtained in the offspring of the isolated heterozygous snails mentioned earlier in this paper. Had haploid parthenogenesis occurred, a ratio nearer to 1:1 would have been expected. If diploid parthenogenesis had occurred exclusively, only pigmented offspring would have been expected. Moreover, the work of Colton (1918) and Crabb (1927b, 1928) indicated that two polar bodies are extruded by the eggs in *Lymnaea*.

Each of the 18 albino snails mentioned above was kept in isolation culture until its death, with one exception. This snail was discarded after producing nothing but albino offspring in its first five egg masses. The ages of the snails at death, in the 14 cases where it was known exactly, varied from 128 to 465 days. The latter figure represents the oldest snail ever reared in this laboratory.

LONGEVITY OF TRANSFERRED SPERM

The time elapsing between the separation of an albino snail from its pigmented mate and the laying of its last "pigment-producing" egg gives an approximate figure for the survival time of transferred sperm in the recipient snail. The maximum time found in this study was 116 days. To get some idea about how fast the fertilizing power of transferred sperm is lost, the data obtained from 13 of the 18 snails referred to earlier were combined. Of the five snails not used in the calculations, three (as mentioned above) had not received sperm from their pigmented mate, and two others died too early to give significant data.

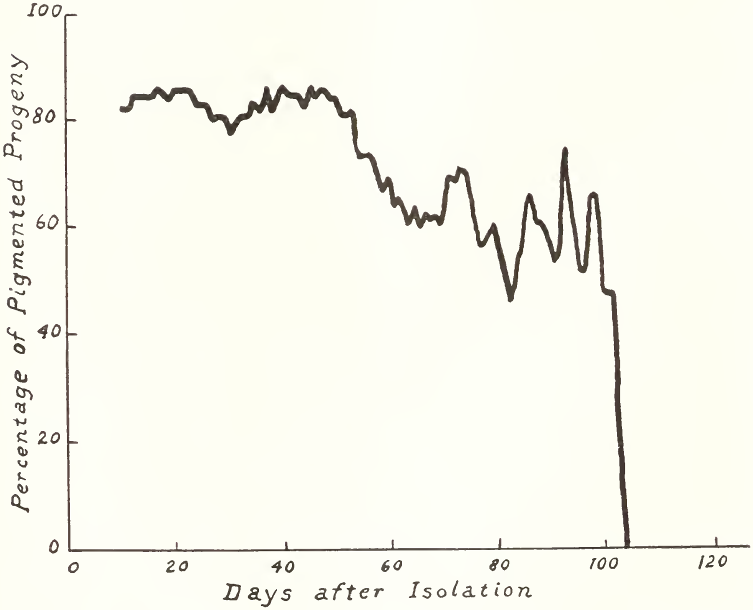

FIGURE 1. Graph showing rate of decrease in percentage of pigmented individuals in the offspring of albino snails that were isolated after receiving sperm from pigmented snails. (Data obtained from egg masses deposited by the albino after the 102nd day of isolation could not be treated in this graph.)

The data from the 13 remaining snails represented 260 egg masses, containing 15,545 eggs. Of these 72.5% hatched; and of those that hatched, 87.8% survived to the "pigment-producing" age. Figure 1 presents a curve showing the percentage of pigmented individuals in the progeny of the 13 snails plotted against time elapsed since separation from their pigmented mates. The curve represents a moving average, smoothed as follows: each point on the curve represents the total pigmented offspring developing from eggs laid in the 10 days just preceding a chosen point, divided by the total offspring produced from all the eggs laid during the same period. The curve thus represents substantially the percentage of cross-fertilization on successive days following separation.

Examination of the curve shows that in the first 50 days cross-fertilization was very high (over 80%), after which time it gradually fell, dropping rather suddenly near the 100th day. As stated earlier the maximum figure obtained was 116 days. Whether this figure really represents the maximum survival period of the sperm or merely the time at which the supply of transferred sperm was all used up, it is impossible to say.

To arrive at a more exact figure for sperm survival after transfer, it would be necessary (1) to add to the figure obtained (116 days) the time elapsing between the last copulation and the separation of the two partners, and (2) to subtract from the figure the time elapsing between actual fertilization and the laying of the egg. These corrections cannot be made from the data here obtained.

Location of Fertilization

The observations of Meisenheimer (1912), Holm (1946) and Abdel-Malek (1954a, 1954b) suggest that the sperm probably enters the egg in or near the "fertilization pocket." Bretschneider (1948a), however, thinks that fertilization may occur as high up in the reproductive tract as the hermaphroditic duct. (The anatomy of the reproductive system of *Lymnaea stagnalis* is shown in Figure 2.)

If foreign sperm after copulation actually travel up the female tract as far as the hermaphroditic duct, as Bretschneider implies, it would seem likely that they would mix with the sperm of the recipient snail. Then if such a mixture of sperm were later transferred in copulation, it is conceivable that some of the foreign sperm might be passed along to a third snail. This possibility was tested as described below.

Ten albino snails that had never been with pigmented snails were paired with pigmented mates until the albinos were seen to function as females in copulation with those mates. Each of these albinos was then marked with finger nail polish on the tip of the shell and placed with another albino which had never been with a pigmented snail. The pairs were maintained until the marked albino was observed functioning as a male in copulation with the second albino. Eggs were saved from the second albino after isolation, and young snails grown from them. In no case were any pigmented offspring obtained.

This negative result indicates either (1) that the transferred sperm did not reach the level of the hermaphroditic duct in any significant number, or (2) that foreign sperm cannot survive a second passage through the reproductive tract in the process of copulation and later movement up the female tract. Since the foreign sperm had already made such a passage once, it seems a bit unlikely that they could

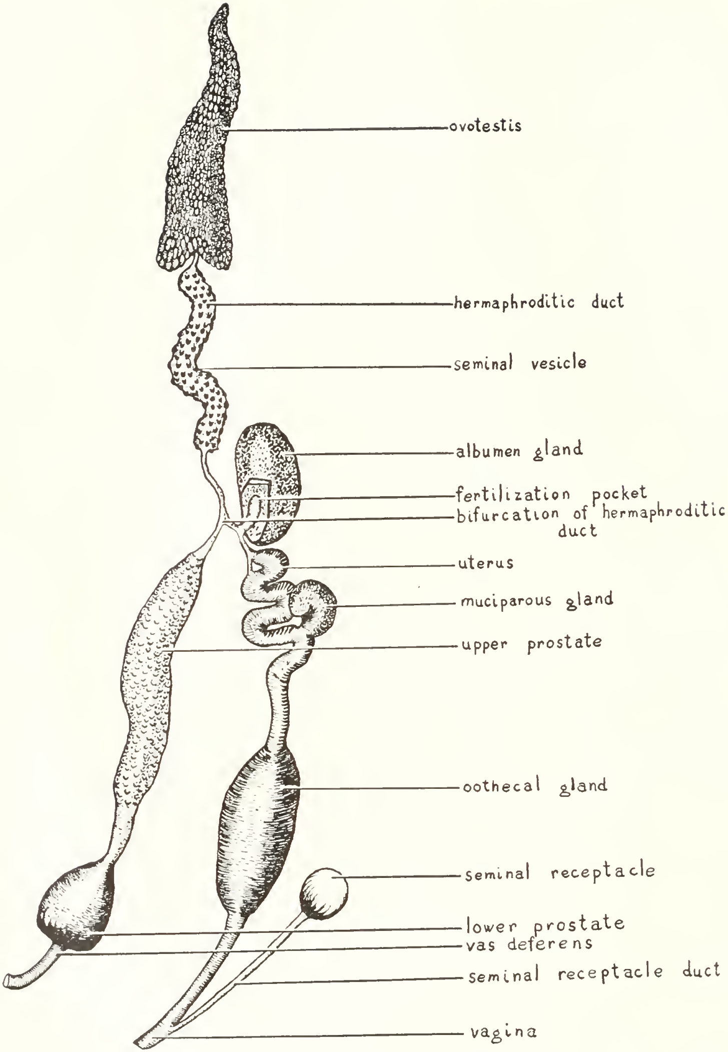
ovotestis
hermaphroditic duct
seminal vesicle
albumen gland
fertilization pocket
bifurcation of hermaphroditic duct
uterus
muciparous gland
upper prostate
oothecal gland
seminal receptacle
lower prostate
vas deferens
seminal receptacle duct
vagina

FIGURE 2. Dorsal view of the reproductive system of *Lymnaea stagnalis appressa* (× 40). The vas deferens has been cut away just beyond the point where it joins with the lower prostate gland. The copulatory apparatus is not shown.

not do it again without injury, though they would undoubtedly be greatly diluted by the other sperm with which they were transferred. These results therefore suggest that foreign sperm probably do not travel up the female tract as far as the hermaphroditic duct.

If, as this suggests, fertilization occurs below the bifurcation of the hermaphroditic duct in the oviduct, it must occur very high up in this latter structure, since the albumen and egg shell is laid down around the egg very soon after the egg enters the oviduct (according to Holm and Bretschneider), and no micropyle has ever been found in the snail's egg shell.

If fertilization does not occur above the point of bifurcation of the hermaphroditic duct, self-fertilization could result only after the transfer of sperm by self-copulation. That self-copulation actually does occur has been observed by many workers. Experiments were made to test this possibility.

Even though snails are extremely difficult subjects for surgical experimentation, the intromittent organ was successfully removed in 9 out of 14 cases. These snails continued to lay eggs after self-copulation was no longer possible. The obvious possibility of prior self-copulation could not be excluded. In several snails in which a section of the vas deferens was experimentally removed without subsequent death of the snail, regeneration re-established a connection. The question, therefore, remains unsettled as to whether prevention of self-copulation will also prevent self-fertilization.

The possibility that the seminal receptacle might serve as an activating organ for the sperm was excluded by examination of seminal receptacles removed from snails at different intervals following copulation. Only in those removed within 30 minutes after copulation were motile sperm found, and the motility was less than that of sperm taken from the vas deferens or ovotestis. The problems of the location of fertilization and the function of the seminal receptacle still remain unsolved.

SUMMARY

1. Albinism in *Lymnaea stagnalis appressa* Say is inherited as a simple Mendelian recessive.

2. Cross-fertilization greatly exceeds self-fertilization in snails allowed to cross-copulate.

3. Transferred sperm may remain viable in the body of the recipient snail for as long as 116 days.

4. It is unlikely that foreign sperm are stored as high up in the reproductive tract as the seminal vesicles, since albino snails previously impregnated by pigmented snails and later mated to virgin albinos engender no pigmented offspring in the latter.

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ACCLIMATION OF OXYGEN CONSUMPTION TO TEMPERATURE IN THE AMERICAN COCKROACH (PERIPLANETA AMERICANA)[1]

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The extensive literature that shows metabolic compensation to temperature among marine poikilotherms (see Dehnel, 1955; Segal, 1955; for reviews) has led us to question the generally reported inability to compensate among insects. Scholander, Flagg, Walters and Irving (1953), having compared certain arctic and tropical insects, find no significant metabolic adaptation to temperature. Edwards (1953) generalizes: he proposes that the metabolic response of insects to temperature can be expressed by a single metabolism/temperature curve. Although several examples of insect acclimation are cited by Bullock (1955) he states (p. 320) that "in spite of these cases, it is believed that insects may be relatively poor in ability to compensate."

Of the well documented cases of insect acclimation to temperature, four are either overwintering or in summer sleep (Lühmann and Drees, 1952; Marzusch, 1952); one is aquatic (Sayle, 1928), and one is in pupa (Heller, 1930). Only a single example concerns an active isolated insect (Parhon, 1909).[4]

We have investigated the American cockroach, *Periplaneta americana,* an insect that is active all year, to see whether or not it behaves similarly to marine poikilotherms with respect to temperature adaptation of the oxygen consumption.

MATERIAL AND METHODS

A culture of nymphal and adult cockroaches was obtained from the Riverside campus of the University of California. This culture had been maintained at 27° C. for a minimum of three generations. Neither molting individuals nor adult females nor individuals under approximately 0.3 gram were used in these experiments. The animals were divided into two groups, nymphs and adults, the former being represented by a wide range of instars. Each of the above two groups was subdivided into three groups of randomly selected individuals. There were fifty animals in each of the six groups. One group of nymphs and one group of adults were placed at 10° C. Similar groups of nymphs and adults were placed at 16°

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[1] This study was conducted at the University of California, Los Angeles. We wish to thank Dr. T. H. Bullock for making the equipment and space available to us.

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[4] Parker (1930) shows acclimation of growth to temperature in two species of insects (*Melanoplus mexicanus* and *Camnula pellucida*). Thompson (1937) finds that embryos of *Melanoplus differentialis* and *Melanoplus femur-rubrum* kept at lower temperatures (20° C.) show a more rapid heart rate than embryos incubated at higher temperatures (30° and 35° C.) when measured at a series of temperatures from 20° to 35° C.

and 26° C. Cockroaches placed at the latter temperature were at essentially the same temperature as the original stock (26°–27° C.); this permitted them to be used as controls.

In order to eliminate the reported light-controlled diurnal activity rhythm (Cloudsley-Thompson, 1953), the animals were maintained in constant darkness. The cockroaches were weighed after each experiment was completed, as it was felt that the added handling of weighing would stimulate the animals to increased activity.

Each of the six groups of animals was given an initial supply of food, and fresh water was added every other day. It was noticed that the cockroaches at 10° C. did not feed. In order to determine whether non-feeding had any effect on the results, a fresh culture was obtained and the experiment was repeated; this time none of the animals was given food.

Oxygen consumption was measured with the use of Wennesland-Scholander microrespirometers which were submerged in a constant temperature bath controlled to ± 0.5° C. The cockroaches were kept at the acclimation (10° and 16° C.) and control (26° C.) temperatures for three weeks. At the end of each week measurements were made on a nymphal sample from each of the three temperature groups. At the end of the first and third weeks similar measurements were made on adult samples from each of the three temperature groups. All the above oxygen consumption measurements were made at 20° C. Those cockroaches from 16° and 26° C. were kept in the 20° C. bath for one hour before measurements were made. Animals from 10° C. were kept at 15° C. for one hour; the temperature of the bath was then raised to 20° C. After one hour at the latter temperature, their oxygen consumption was measured.

In addition to the above experiments, two samples of nymphs were separated from the original culture (26° C.). The oxygen consumption of those from the first sample was measured over a descending series of temperatures (30°, 25°, 20°, 15° and 10° C.). The first sample was then placed at 26° C. and the other at 10° C. At the end of three weeks the oxygen consumption of animals from the first sample was measured over the same descending series of temperatures. The oxygen consumption of animals from the second sample was measured over an ascending series of temperatures (10°, 15°, 20°, 25° and 30° C.).

Measurements for all experiments were made at fifteen-minute intervals for a period of one and one-half to three hours. On all figures each point represents one animal. The coordinates are log-log and the curves are eye-fitted.

RESULTS

Nymphs. When the oxygen consumption of equal weight animals kept at 10°, 16° and 26° C. is measured at 20° C., it is found that those animals maintained at the lower temperatures show the higher consumption (Fig. 1). The increase in oxygen consumption of animals kept at 10° and at 16° C. occurs within the first week. For the duration of the experiment no further increases were observed. Under the conditions of this experiment it is impossible to compare the time required for acclimation in the two groups; it can be said only that in both it is complete within one week. Because there is no difference in the weekly oxygen consumption values for each group, they are combined for the regression curves in Figure 1.

For purposes of comparison within the three groups of cockroaches, animals with an average weight of 0.6 gram were chosen from the regression curves. This weight was chosen because it falls approximately within the center of the weight range on each of the regression curves. When the oxygen consumption of this 0.6-gram animal is read directly from the graph it is noted that (1) this weight animal acclimated to 10° C. consumes 67 mm.^{3}/gm./hr. (57%) more oxygen than his counterpart acclimated to 26° C., (2) this weight animal acclimated to 16° C. consumes 42 mm.^{3}/gm./hr. (36%) more oxygen than his counterpart from 26° C. and (3) this weight animal from 10° C. consumes 25 mm.^{3}/gm./hr. (16%) more oxygen than his counterpart from 16° C. It is apparent that the animals acclimated

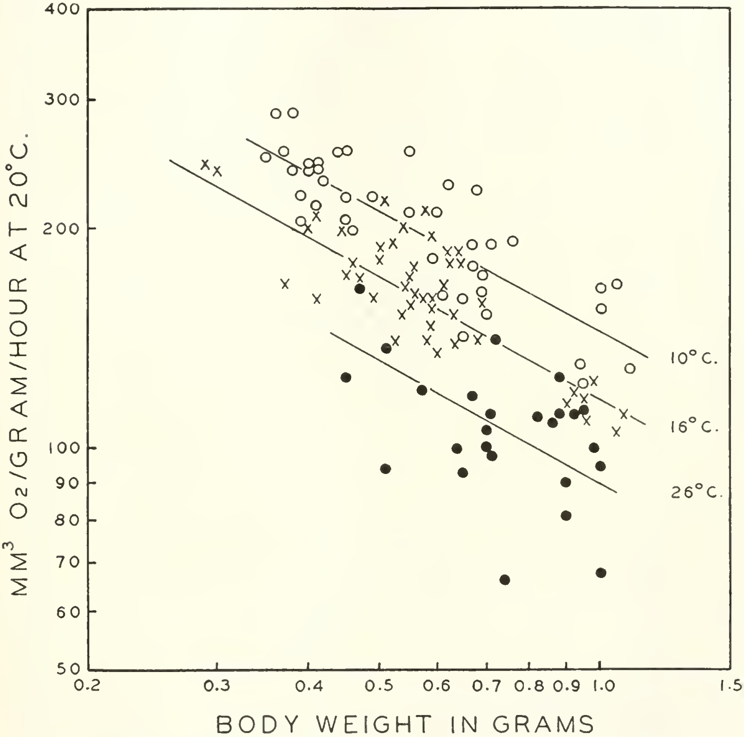

FIGURE 1. Weight-specific oxygen consumption as a function of weight in nymphal *Periplaneta americana*. Animals were kept at 10°, 16° and 26° C. for one to three weeks, and the measurements were made at 20° C. In all figures each point represents the average oxygen consumption for one animal over a period of one and one-half to three hours. Open circles represent 10° C. animals, crosses, 16° C. animals and closed circles, 26° C. ones. The coordinates are logarithmic, and all curves are eye fitted. Results from feeding and non-feeding experiments are combined.

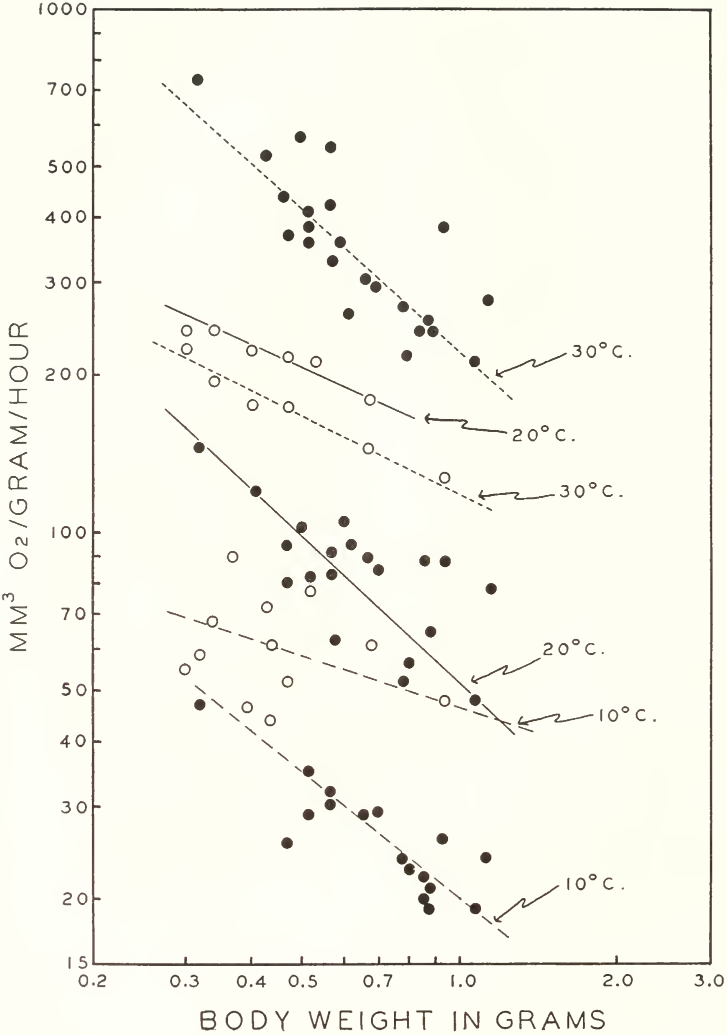

FIGURE 2. Weight-specific oxygen consumption as a function of weight in nymphal *Periplaneta americana* measured over a series of temperatures. Open circles represent cold-adapted animals (10° C.); closed circles represent warm-adapted animals (26° C.).

to 10° C. are responding to the increased distance (° C.) from the control temperature (26° C.) with a further increase in oxygen consumption. The values indicate that a linear relation exists between the increase in oxygen consumption and the decrease in acclimation temperature (4.2 mm.^{3} O_2/gm./hr. increase per degree centigrade drop in temperature).

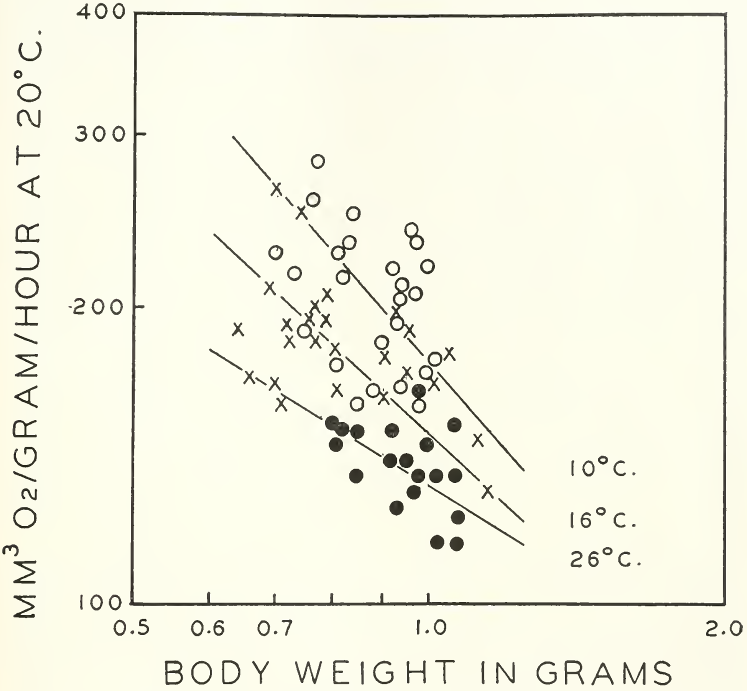

FIGURE 3. Weight-specific oxygen consumption as a function of weight in adult *Periplaneta americana.* Animals were kept at 10°, 16° and 26° C. for one to three weeks, and the measurements were made at 20° C.

Nymphs, rate/temperature experiment. The results of the rate/temperature experiment are presented in Figure 2. The rate values obtained at 15° and 25° C. are omitted to make the graph easier to read. From 10° to 25° C. cold-acclimated nymphs (10° C.) consume more oxygen per gram per hour than equal weight warm-acclimated nymphs (26° C.). The oxygen consumption of the cold-acclimated nymphs is depressed at 30° C., *i.e.,* less oxygen is consumed at 30° C. than at 20° C. Since the curves at 30° and at 20° C. are parallel, the oxygen consumption of large and small nymphs is depressed equally.

At all temperatures the regression lines for the warm-acclimated nymphs are steeper; the regression lines at the different temperatures for either the warm- or cold-acclimated animals are essentially parallel.

If the curves (Figs. 1 and 2) representing nymphs acclimated to 10° C. and measured at 20° C. are compared, it is seen that the slopes and positions of the curves are the same. However, if a similar comparison is made for the warm-acclimated nymphs, it is found that the oxygen consumption is constantly lower for these animals in the rate/temperature experiment. These animals spent approximately four hours at 30° C., before they were measured at 20° C. It is possible that four hours is sufficient time for the acclimation process to have begun. Therefore, the oxygen consumption at 20° C. is lower than it is for the animals brought directly from 26° to 20° C.

Adults. Adult cockroaches, like nymphal cockroaches, show acclimation of their oxygen consumption to temperature. Those adults kept at 10° and 16° C. consume more oxygen per gram than do equal weight adults kept at 26° C. when all are measured at 20° C. (Fig. 3). As with the nymphs no change was found in the weekly (first and third) oxygen consumption values for each temperature group. Therefore, these values are combined for each of the regression curves in Figure 3.

For the adult cockroaches, animals with an average weight of 0.9 gram were chosen. When the oxygen consumption of this 0.9-gram adult is read from the graph it is noted that (1) this weight animal acclimated to 10° C. consumes 55 $mm.^3/gm./hr.$ (40%) more oxygen than his counterpart acclimated to 26° C., (2) this weight animal acclimated to 16° C. consumes 25 $mm.^3/gm./hr.$ (18%) more oxygen than his counterpart from 26° C. and (3) this weight animal from 10° C. consumes 30 $mm.^3/gm./hr.$ (18%) more oxygen than his counterpart from 16° C. These values suggest that in contrast to the nymphs, a non-linear relationship exists between the increase in oxygen consumption and the decrease in acclimation temperature (2.5 $mm.^3$ $O_2/gm./hr.$ increase per degree centigrade drop in temperature from 26° to 16° C.; 5.0 $mm.^3$ $O_2/gm./hr.$ increase per degree centigrade drop in temperature from 16° to 10° C.).

Comparison of nymphs and adults. Comparison of the oxygen consumption of nymphal and adult cockroaches (Figs. 1 and 3) that have been acclimated to and measured at the same temperatures shows that adult cockroaches consume more oxygen per gram than equal weight nymphs. Although the adult curve representing the control animals (26° C.) is displaced above the curve for the control nymphs (26° C.), the slopes are essentially parallel. With acclimation to 16° and to 10° C. small and large nymphs respond in a like manner and these curves have approximately the same slopes as the 26° C. curve. On the contrary, small and large adults show a differential response to the temperatures of acclimation (with extrapolation, the curves in Fig. 3 would intersect to the right). Small adults are responding to the decreased temperatures of acclimation with a greater increase in their weight-specific oxygen consumption than are large adults. Small adults are therefore doing a better job of acclimating than large adults and all sizes of nymphs are doing a better job than all sizes of adults.

DISCUSSION

The object of this investigation was to see if the insect *Periplaneta americana* could acclimate its metabolic activity to temperature. However, we would first like to discuss an additional observation. We have found that adult cockroaches are living at a faster metabolic pace than are nymphs of approximately the same weight (see Figs. 1 and 3). Batelli and Stern (1913) showed that at all temperatures from 20° to 40° C. fly imagines consume more oxygen per unit body weight than did larvae. Similarly, it was found by Ludwig (1931) that the weight-specific oxygen consumption of adult Japanese beetles, *Popillia japonica,* was greater than that of the larvae. Referring to the Holometabola, Wigglesworth (1950, p. 413) has stated that "metabolism at a given temperature is generally much higher in the adult than in the larva and higher in the larva than in the pupa." Wigglesworth attributes this difference to the increased activity metabolism of the adult. Many investigators will speak of the typical "U-shaped" respiratory curve during the metamorphosis of holometabolus insects (see Edwards, 1953, for references).

The available data for the Hemimetabola (the insects in which there is little or no change in shape during ontogeny) are much less than for the Holometabola. Edwards (1953) presents a curve showing the change in weight-specific oxygen consumption from egg deposition through early adulthood of the milkweed bug *Oncopeltus fasciatus.* Within a few days after the last molt, adults consume more oxygen per gram per hour than do last instar nymphs. If the oxygen consumption during molt is ignored, then the curve resembles a flattened "U-shape." In this study early adult and late nymphal *Periplaneta americana* show a similar relationship. With increasing size of the adult, the characteristic fall in weight-specific oxygen consumption is observed.

We do not know what makes possible the elevated metabolic activity of the early adult cockroaches; we have not observed a difference in locomotor activity that would account for it. Perhaps the elevation in rate is a consequence of the metamorphosis from nymph to adult. A similar suggestion was offered by Groebbels (1925) to account for the increase in metabolic rate found during metamorphosis of *Rana* tadpoles.

Contrary to the generalized statement of Edwards (1953) that insects do not compensate metabolically to temperature, both nymphs and adults of *Periplaneta americana* adapted to 10° C. consume more oxygen, per animal and per gram, than equal weight control animals adapted to 26° C. when measured at the same temperature.

Lühmann and Drees (1952) and Marzusch (1952) show temperature adaptation in four species of insects, two of which are overwintering (the potato beetle, *Leptinotarsa decemlineata,* and the leaf beetle, *Phytodecta rufipes*) and two in summer sleep (the potato beetle, *Melasoma populi,* and the leaf beetle, *Galeruca tanaceti*). These investigators are unable to show temperature adaptation during the active feeding period. Lühmann and Drees have suggested that the compensatory response is masked by the high metabolic activity associated with feeding. We believe that if such a response can be demonstrated at any given time, it does seem reasonable to expect this ability to be present at all times. This expectation is borne out by *Periplaneta americana,* which is active and feeds all year. Therefore, it is difficult to understand why this compensatory response appears only in these insects under conditions of winter and summer sleep. It would be well to note that one species of leaf beetle (*Chrysomela haemoptera*) shows no adaptation even though its metabolic level was depressed during the summer sleep (Lühmann and Drees, 1952).

Previously, cold- and warm-adapted groups have been compared at a given temperature or between temperatures by arbitrarily choosing a weight and determining the oxygen consumption for each group. It is also profitable to choose an arbitrary rate of oxygen consumption and determine the approximate weight of animal in each group for which this rate is obtained (Figs. 1 and 3). As a generalization, a large cold-adapted cockroach consumes about as much oxygen as a small warm-adapted one. For example, a 0.4-gram nymph (26° C.), a 0.7-gram nymph (16° C.) and a 0.9-gram nymph (10° C.) when measured at 20° C. consume equal amounts of oxygen per unit weight. Similarly, it is possible to determine the temperatures at which cold- and warm-adapted roaches consume the same amount of oxygen (Fig. 2). On this basis, cold-adapted animals consume at 15° C. slightly more oxygen than warm-adapted animals consume at 20° C.

Sayle (1928) tested the effect of low temperature on carbon dioxide production of dragon fly nymphs (*Aeschna umbrosa*). She lowered the temperature from 22° C. to 13° C. (three days at 17° C. and three days at 13° C.) and found that carbon dioxide production was about the same at the lower temperature as the initial production at 17° C. after the first day. The major portion of acclimation of these nymphs was evident within forty-eight hours. It is not unreasonable to expect that the rate of acclimation in *Periplaneta americana* is equally as rapid since no further change was evident after six days. In addition, animals measured at 20° C. after spending a number of hours at 30° C. consume less oxygen than animals measured at 20° C. directly from 26° C. Such time courses as found in these animals compare favorably with that shown for other species (Behre, 1918; *Planaria dorotocephala;* Roberts, 1952, *Pachygrapsus crassipes;* Segal, 1955, *Acmaea limatula*).

Bullock (1955) has thoroughly reviewed the known cases of acclimation to temperature at the several levels of organization (molecular, cellular, tissue and organ system). He does cite several negative instances in which animals fail to show acclimation. However, the evidence from widely divergent groups, involving different physiological systems, suggests to us that compensatory responses to environmental stresses are inherent components of protoplasmic systems. Negative cases as cited by Bullock (1955) do not invalidate this idea. Such instances suggest to us that animals, in which no acclimation was found in the particular physiologic system studied, might show compensation to stress in another system or at a different level. Compensatory responses to temperature are most often described, but other environmental parameters (osmotic pressure, drugs, oxygen tension; see Prosser, 1955) equally as important may evoke such adaptation. If this phenomenon is a universal component of living systems and permits animals to assume degrees of environmental independence, it goes far to explain their survival and distribution. Within limits it accomplishes the same results as homiothermism accomplishes for the warm blooded animals.

SUMMARY

1. Oxygen consumption has been studied in cultures of nymphal and adult cockroaches, *Periplaneta americana,* that have been maintained at two experimental temperatures (10° and 16° C.) and the control temperature (26° C.) for a period of one to three weeks.

2. It has been shown that the oxygen consumption of equal-weight nymphs when measured at 20° C. is higher in animals that have been maintained at the lower temperatures.

3. Comparison of cold- (10° C.) and warm-adapted (26° C.) nymphs when measured at a series of temperatures (10° to 25° C.) demonstrates that cold-acclimated animals consume more oxygen per gram per hour than equal weight warm-adapted ones.

4. Adult cockroaches show acclimation of their oxygen consumption to temperature. However, there is a differential response with respect to size; small adults acclimate to a greater degree than large ones. Further, all sizes of nymphs show a greater degree of acclimation than all sizes of adults.

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NEUROSECRETORY CELL TYPES AND THEIR SECRETORY ACTIVITY IN THE CRAYFISH[1, 2]

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It is now well known that physiologically active substances are produced in neurosecretory cells located throughout the nervous systems of crustaceans (Bliss, 1951, 1952, 1953; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Carlisle, 1953; Enami, 1951; Passano, 1951a, 1952, 1953). Furthermore, the neurosecretory cells are distributed as distinct groups (Bliss, Durand and Welsh, 1954; Enami, 1951), at least in the eyestalk and brain. Relatively little is known about the specific localization of the sources of the neurohormones affecting particular physiological processes; however, Passano (1951a, 1951b, 1952, 1953) has shown that the x-organ in crustaceans produces a substance that is capable of inhibiting molt.

Neurosecretory cells have been described for the x-organ (Bliss, 1952; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Carlisle and Passano, 1953; Enami, 1951; Passano, 1953); but, with the exception of Enami's work on *Sesarma* (1951), there is little information concerning the different types of neurosecretory cells present in crustaceans. Furthermore, there is no cytological evidence available to indicate which of the different neurosecretory cell types are involved in the physiology of molt. It is apparent that work along these lines is needed, particularly in view of the fact that cytological differences in cell types often go hand in hand with differences in function.

The present paper will be concerned with a histological study of the neurosecretory system of the crayfish, *Orconectes virilis* (formerly *Cambarus virilis*) in relation to the molting cycle.

MATERIALS AND METHODS

1. *Animals*

The animals used in this study were mature males, approximately five centimeters in carapace length, all collected from Hobb's Brook Reservoir, Lincoln, Mass., in the summer of 1954. Mature crayfish were collected on the dates shown in Table I. With the exception of May animals, which had been kept in the laboratory for three to four months and fed weekly on clam and fish, eyestalks and brains were removed and fixed on the same day the animals were collected.

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[1] This work constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from Harvard University. The writer wishes to express his sincere thanks to Professor John H. Welsh under whose direction this work was carried out.

[2] The preparation of the manuscript was aided by a grant from the Research Council, Rutgers University.

2. *Dissections*

All dissections were performed in crayfish perfusion fluid (van Harreveld, 1936). A pair of fine iridectomy scissors, jeweler's forceps and cuticle scissors were used in the dissections.

Eyestalk. The eyestalk was removed by cutting the pedunculus lobi optici with a pair of small cuticle scissors. Next, the chitinous exoskeleton was cut the full length of the eyestalk on each side. The eyestalk was then pinned ventral side down by means of size 0 insect pins in a Syracuse watch glass, half filled with paraffin and containing crayfish perfusion fluid. The remainder of the dissection was carried out with the aid of a binocular dissecting microscope.

After a cut was made across the dorsal half of the retina, the proximal end of the top half of the exoskeleton was lifted and the hypodermis was carefully scraped from the exoskeleton. Great care was taken in this step to prevent excess stretching of the nerve tissue.

The cut end of the pedunculus lobi optici was grasped with fine forceps, and the eyestalk contents were separated from the underlying exoskeleton. The whole content of the eyestalk was then placed in a vial containing fixative. With practice, this procedure could be accomplished within two to three minutes. Excellent fixation was obtained in all cases.

Brain. The head of the animal was removed by a cut just posterior to the brain and mouth. The exposed parts were immediately rinsed thoroughly with perfusion fluid to remove any stomach contents, pieces of hepatopancreas, or urine released after puncture of the bladders. Frequent changes of the perfusion fluid were made throughout the dissection. The rostrum of the animal was next inserted in a piece of modeling clay in such a manner that the open end of the head was facing up. In this way, the animal's head served as a miniature dissecting vessel. The remainder of the procedure was carried out with the aid of a dissecting microscope.

After removal of the stomach, pieces of hepatopancreas, and green glands, the brain was rinsed thoroughly with perfusion fluid. All nerves leading from the brain and the connective tissue sheath surrounding the brain were cut away, and the brain was placed in a vial containing fixative. The brain was lifted by means of the circumoesophageal connectives. This procedure required about three to four minutes.

3. *Histological procedure*

The fixatives employed in this study were Helly's fluid (fixing time, eight hours) and Bouin's plus one per cent calcium chloride (fixing time, twenty-four hours). Tissues were dehydrated in alcohol, cleared in cedar oil and embedded in Tissuemat (melting point 56–58° C.). Sections were cut at 6 μ and stained with aldehyde fuchsin (Gomori, 1950) according to the schedule of Halmi (1952), but with modifications by Dawson (1953). This procedure involved a permanganate oxidation prior to staining and will be referred to in the text as PAF. Sections were also stained with chrome-alum-hematoxylin-phloxin (Gomori, 1941) as adapted by Bargmann (1949). This technique is referred to in the text as CHP.

4. *Cell counts*

A study of cell types revealed that secretory material was present as small granules or droplets within the cells. The secretory activity of a group of cells could be judged, therefore, by counts of cells which appeared histologically to be in a given stage of the secretory cycle.

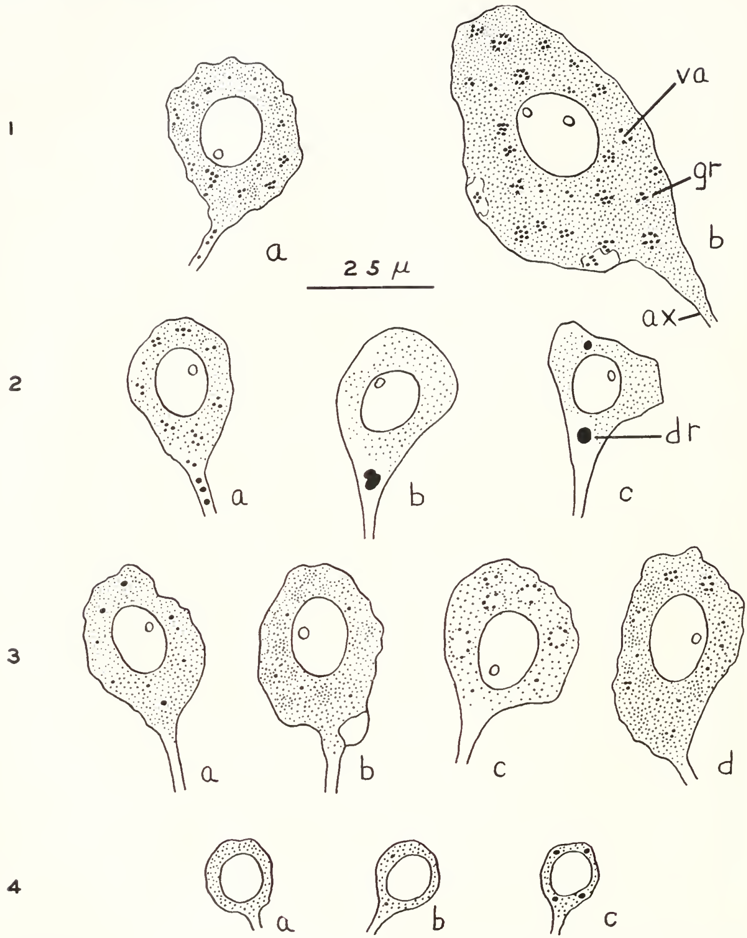


FIGURE 1. Drawings of neurosecretory cell types in the eyestalk and brain of the crayfish. Numbers along the left column indicate cell types. Letters indicate cells in successive stages of the secretory cycle. ax, axon; dr, droplet; gr, granule; va, vacuole.

Type 1 cells of the x-organ, in a stage of the secretory cycle similar to that shown in Figure 2, were counted. These cells are large enough so that they can be recognized from section to section and were counted only when the nucleus was included in the section. In this way no cell could be counted twice.

Type 2 cells in the x-organ were also counted. In this case, cells which contained both a nucleus and a secretory droplet (Fig. 1, Cells 2b, c) in the same section were counted. The nuclei of these cells are small enough so that a section near the center of the nucleus would be present only once per cell. This method of counting resulted in minimum counts of the cells in that particular stage of the secretory cycle. Type 2 neurosecretory cells as shown in Figure 1, Cell 2a, were not counted. The marked uniformity of cell counts during all months except May and June indicates that consistent results can be obtained in this manner.

This method of counting could not be applied to the other neurosecretory cell groups because the secretory material in those groups is freely distributed throughout the cytoplasm in the form of fine granules (see below).

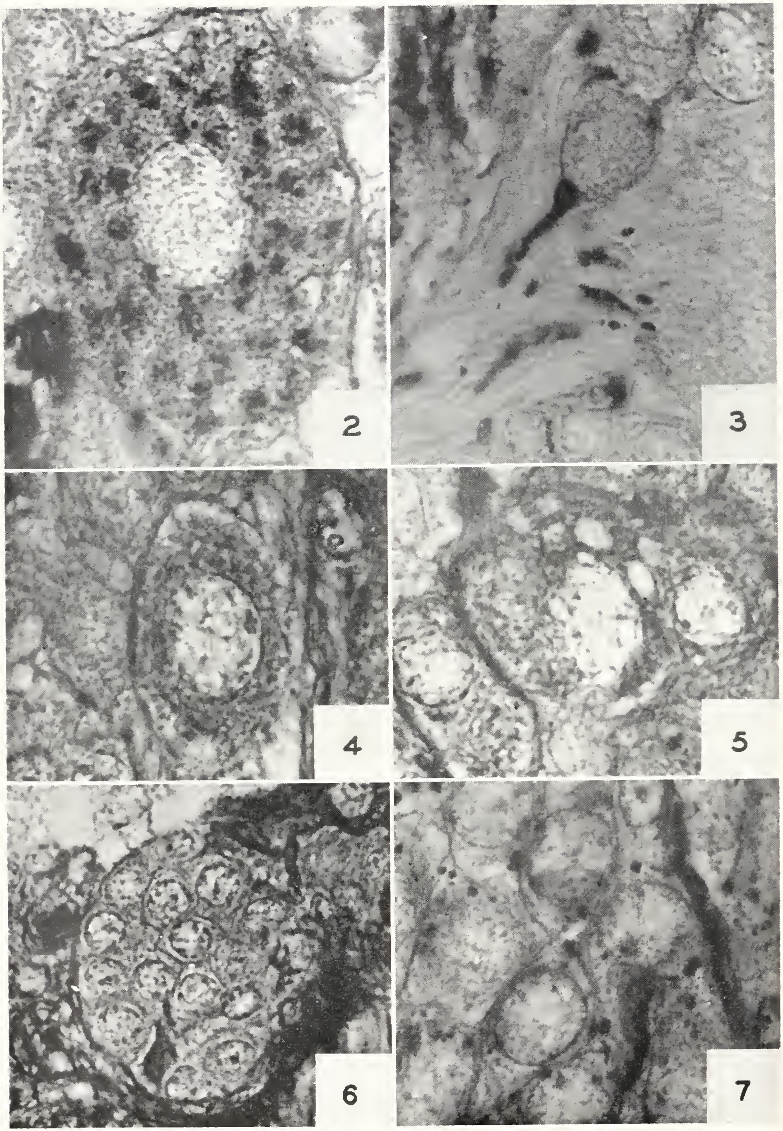
Results

Studies of serial sections of eyestalks and brains, stained with CHP and PAF, have revealed the presence of large groups of cells (see also Bliss, Durand and Welsh, 1954) that are histologically different from the hundreds of ordinary ganglion cells present throughout the eyestalk and brain. These cells are always larger than the ordinary ganglion cells. Most of them possess large nuclei, have abundant cytoplasm, and are characterized by the presence in the perikaryon and axon of droplets of a material which stains conspicuously with aldehyde fuchsin and chrome-hematoxylin. Not all the cells have the same appearance as to the quantity and size of these droplets. These characteristics lead to the conclusion that the cells are neurosecretory cells as defined by E. Scharrer and B. Scharrer (1945) and described in a great variety of animals by numerous authors (see especially Scharrer and Scharrer, 1954; Gabe, 1954).

There appear to be four neurosecretory cell types (Fig. 1) found in the eyestalk and brain of the crayfish, *O. virilis*. Size, general shape of the cell body, presence or absence of vacuoles in the cytoplasm and the appearance of the secretory product were used as the main criteria in separating the cell types. Since large numbers of the cells were found to form more or less distinct subdivisions of larger units in the case of two cell types, and possessed a fairly uniform set of the characteristics listed above, it is believed that the cells are truly of different types and have not been confused with various stages of the secretory cycle present in a given cell type. The only cells that others might possibly find difficult to recognize are those similar to Type 1 (a) and Type 3 (d) (Fig. 1).

Cell types

Cell Type 1. The distribution of this cell type is somewhat limited; it is most numerous in the x-organ and lies as a distinct subgroup in the most distal portion of the x-organ. The cell bodies are large, 40–60 μ in length, possess much cytoplasm and contain a large nucleus, 15 μ in diameter. In the material used in this study, Type 1 cell bodies have extremely irregular outlines which are very likely caused by shrinkage during fixation (Fig. 1). The nucleus may often contain two and some-



Figures 2–7.

times three nucleoli. Moreover, nucleoli are usually peripheral, lying against the nuclear membrane. Further, not all of the cells show the presence of large amounts of secretory material at any one time. This, however, is to be expected, for apparently some cells are at the peak of their secretory processes while others are in a quiescent state. The secretory product consists of a great number of aggregations of small (0.5–1.0 μ) granules that stain with aldehyde fuchsin (Figs. 1 and 2). It frequently appears as though the aggregations are located on the surface of small clear spaces in the cytoplasm. In cells that do not contain large amounts of secretory material, aggregations may not be present. In these cases secretory material is scattered in the cytoplasm as fine granules about the size of those that make up the aggregations. The cytoplasm is generally flaky in appearance and, in cells containing many granules, may sometimes be stained a red-purple by the PAF technique. However, many of the cells do not show this cytoplasmic staining; this is probably because the cells are in different stages of the secretory cycle.

In some sections, secretory material may also be seen at a point where the axon leaves the cell body and along the axon for a short distance. When it is found along the axon, the secretory material appears as a number of small granules strung out along the axon. Farther from the cell bodies, though, it appears to consist of a more finely divided suspension somewhat dispersed in the axons.

Cell Type 2. This cell type is a smaller cell which is also restricted in its distribution. These cells are arranged in the proximal part of the x-organ as a cluster of grapes as described by Hanström (1931). The cell body measures about 30 μ in length and is slightly narrower, 20–25 μ, than it is long (Fig. 1). It possesses a large nucleus, but none has been observed to contain more than one nucleolus. The nucleolus here is also near the nuclear membrane. The cytoplasm is somewhat vacuolated, although the vacuoles appear to be a result of fixation; they do not possess any definite shape. In February, these cells possess small vacuoles with granules of secretory material located peripherally. Some cells in February have granules contained within vacuoles. At other times of the year the material is present as quite large, 4 μ, distinct droplets (Figs. 1 and 9) as contrasted with the granules present in Type 1 cells. The droplets, usually one or two per cell, are almost always round and are usually located in the axon hillock or in the axon. Sometimes many drops may be seen along the bundles of axons as they leave the cell group (Fig. 3).

FIGURE 2. Type 1 neurosecretory cell in advanced stage of the secretory cycle. Note the aggregations of granules. Cells with this appearance were counted as indicating the secretory activity of this cell type. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin, 1300 ×.

FIGURE 3. Type 2 neurosecretory cell containing many droplets of secretory material in its axon hillock. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1300 ×.

FIGURE 4. Type 3 neurosecretory cell showing peripheral arrangement of vacuoles (top of photograph). Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1300 ×.

FIGURE 5. Type 3 neurosecretory cell. Note centrally located vacuole with granules of secretory material located on the surface. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1400 ×.

FIGURE 6. Group of Type 4 neurosecretory cells in the eyestalk. Note scanty cytoplasm and scarcity of secretory material. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 700 ×.

FIGURE 7. Group of Type 4 neurosecretory cells in the brain. Note large content of secretory material. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 1200 ×.

Cell Type 3. This cell type is distributed freely throughout the neurosecretory cell groups in the eyestalk and brain with the exception of the x-organ. These cells are, on the average, slightly larger than the Type 2 cells (Figs. 1, 4 and 5). They are generally tear-drop shaped, although not as distinctly so as the Type 2

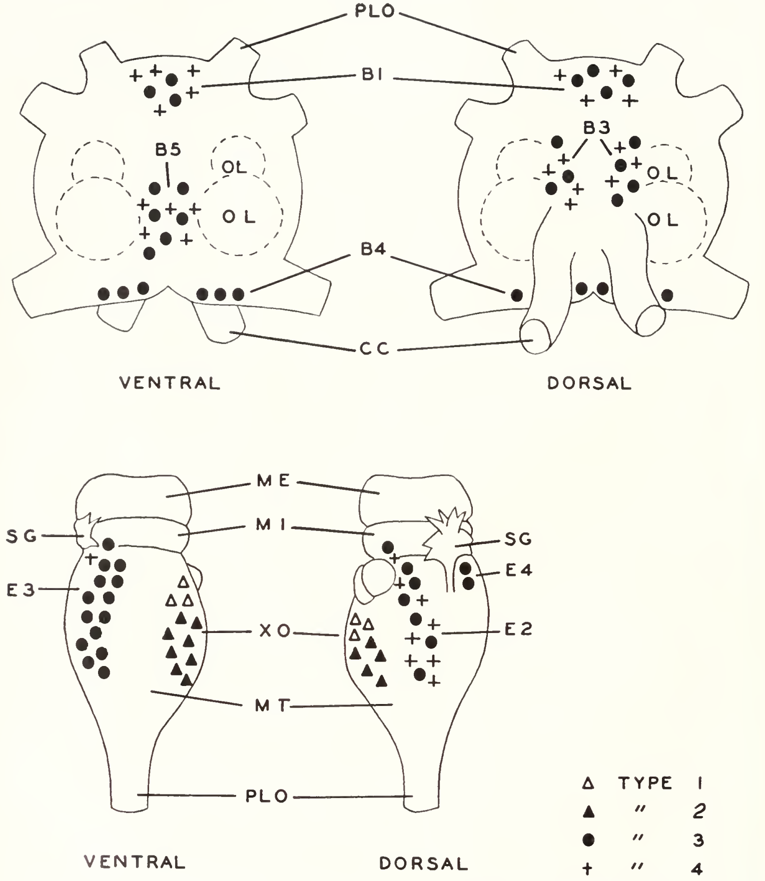

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FIGURE 8. Diagrammatic representation of the distribution of neurosecretory cell types in the brain and eyestalk of the crayfish. Compare with Enami's Figure 11 (1951). B1 through B5 designate groups of neurosecretory cells in the brain. E1 through E4 designate groups of neurosecretory cells in the eyestalk. CC, circumoesophageal connectives; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OL, olfactory lobes; PLO, pedunculus lobi optici; SG, sinus gland; XO, x-organ.

cells. Two characteristics distinguish these cells from the Type 2 cells. The first characteristic is the nature of the vacuoles. The vacuoles are rather large, up to 7 μ, and most of the time may be seen around the periphery of the cell (Figs. 1 and 4) although sometimes they may be located more centrally in the cytoplasm (Figs. 1 and 5). The vacuoles are sharply delimited from the cytoplasm. In this way they are markedly different from those usually found in Type 2 cells. The second characteristic is the appearance of the secretory product. Thus, in the Type 3 cells, the secretory product consists of fine granules which are never clumped in as large numbers as they are in the Type 1 cells (Fig. 2). Furthermore, secretory material is never present in the form of large droplets as it is in Type 2 cells. Granules are scattered, apparently at random, throughout the cytoplasm or they may be found on the surface of vacuoles or, sometimes, as a small drop in the center of one of the vacuoles (Fig. 1). Vacuolated cells of this type are sometimes found to contain no signs of secretion.

Cell Type 4. Cells of this type are located in all neurosecretory cell groups of the eyestalk and brain except the x-organ and group E3 (Fig. 8). The Type 4 cells are small, about 13 μ in diameter, possess a small nucleus, 10 μ in diameter, and, as is obvious from the measurements, very little cytoplasm (Figs. 1 and 6). They are classified as neurosecretory cells since preliminary studies show that, under certain conditions, some of the cells undergo changes in the amount of secretory material they contain (Fig. 7). Furthermore, they are similar to the gamma neurosecretory cells described by Enami (1951) and are found only within the neurosecretory cell groups. Generally they show little sign of secretory activity but differ from the ordinary ganglion cells of the eyestalk in that they possess more cytoplasm and cell boundaries which are easily demonstrated by the techniques used in these studies. The boundaries of the ordinary ganglion cells are extremely difficult to detect with these techniques.

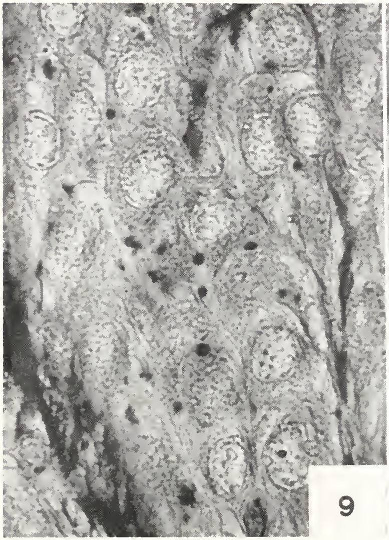
The distribution of neurosecretory cell types is shown in Figure 8. It should be noted that certain cell groups of the crayfish differ in their distribution from that reported in a previous account (Bliss, Durand and Welsh, 1954). The earlier account is essentially correct. However, groups B2 and B3 of the earlier account most likely constitute one group of cells. The group was previously reported to lie lateral to the olfactory lobes. Actually it is located medial to the olfactory lobes on the lateral side of the main mass of fibers of the brain. This distribution of neurosecretory cells brings the neurosecretory system of the crayfish into fairly close agreement with that of *Sesarma* (Enami, 1951).

Secretory activity

No published observations on the normal molting cycle of *O. virilis* are available. However, the following information, although incomplete, shows that there is a single molting time per year for crayfish of the size and sex used in this study. All animals collected on June 28 were soft; the cuticle was parchment-like. Considerable resorption of calcium had occurred from all parts of the exoskeleton and especially from the ischiopodite of the cheliped. Further, all of the animals possessed well developed gastroliths, about 3 mm. in diameter, contained within the gastrolith sac.

Similarly, all animals collected on July 23 were soft; their exoskeletons were thin and parchment-like, but none possessed any signs of gastroliths. All animals collected on August 14 had hard exoskeletons and showed no signs of an approaching molt.

Since the gastroliths disappear very shortly after molt, the observations indicate that these animals had molted some time between June 28 and July 23. Furthermore, the observations show that the adult male animals used in this study were highly synchronized in their molting period, for none appeared to be approaching a molt on any date after June 28.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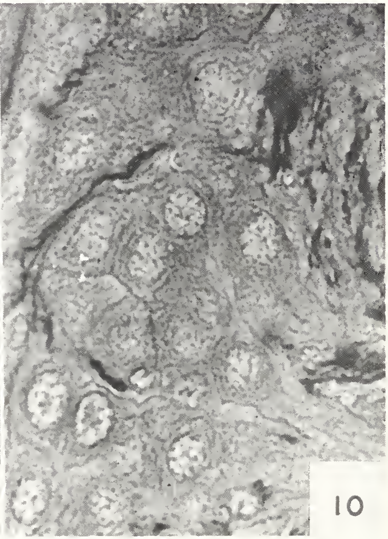
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FIGURE 9. Type 2 neurosecretory cells in the x-organ of a crayfish just prior to molt. Note the large number of secretory droplets. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 700 ×.

FIGURE 10. Type 2 neurosecretory cells in the x-organ of a crayfish shortly after molt. Note the lack of secretory material. Bouin's plus 1% calcium chloride; permanganate-aldehyde-fuchsin; 700 ×.

For the greater part of the year, the number of cells that contained secretory material was remarkably constant. However, a striking increase in the number of Type 2 cells that contained droplets of secretory material took place some time before May 6. From Table I it will be observed that more than twice as many Type 2 cells contained secretory droplets on May 6 and June 28 (Fig. 9) than at any other time of sampling (Fig. 10).

Table I shows that the number of Type 1 cells that contained secretory material did not change appreciably throughout the year.

The other neurosecretory cell groups in the eyestalk were examined carefully, but no apparent histological changes occurred in these secretory cells during the year. The scarcity of secretory material, relative to the amounts present in cell Types 1 and 2, and its occurrence as small granules made it difficult to determine what proportion of cells showed secretory activity. It was concluded, however, that no major histological changes occurred in the other neurosecretory cell groups in the course of this study.

DISCUSSION

Cell types

A few comments should be made regarding a comparison of the neurosecretory cell types of the crayfish with those described for other crustaceans by Enami (1951), Matsumoto (1954), and Carlisle and Passano (1953). Although these authors studied brachyurans and used fixatives other than those used by the present writer, their findings bear similarities with those reported here for the crayfish.

TABLE I

Counts of Type 1 and Type 2 neurosecretory cells containing secretory material in the brain and eyestalk of Orconectes virilis

Date	Number of animals	Cell type	Mean count and standard error
May 6	2	2	117±1
June 28	4	1	43±1
	4	2	125±8
July 23	4	1	46±2
	4	2	53±5
August 14	3	1	51±2
	3	2	66±4
August 31	4	1	38±2
	4	2	38±4
September 22	3	1	40±1
	3	2	38±6

Of the four neurosecretory cell types described above for the crayfish, it has been shown that the Type 1 and Type 2 cells are practically restricted in their distribution to the x-organ; only a few Type 1 cells are found in other neurosecretory cell groups. A study of Enami's figures reveals that in *Sesarma,* the giant beta neurosecretory cell is the only type found in the x-organ of that animal. Smaller beta neurosecretory cells are present as a small paired group in the supraoesophageal ganglion of *Sesarma.* Enami reports that the cytoplasma of the beta neurosecretory cells is fairly homogeneous and of compact appearance, showing but slight contraction upon fixation. It is apparent from other figures in Enami's paper that the Type 2 cells in the crayfish are similar to the giant beta cells in *Sesarma.*

No Type 2 neurosecretory cells were found in the brain of the crayfish. The crab, *Sesarma,* would appear to differ from the crayfish in that the crab possesses a paired group of beta neurosecretory cells in the supraoesophageal ganglion. In addition, Enami describes no cells in *Sesarma* which are comparable to the Type 1 cells of the crayfish.

The Type 3 cells of the crayfish are comparable to the alpha cells of *Sesarma* in their distribution and in some of their cytological details. In both animals they are found in all neurosecretory cell groups except the x-organ. Both cell types are rich in cytoplasm and are characterized by the presence of vacuoles which are sharply delimited from the cytoplasm.

The Type 4 neurosecretory cells of the crayfish are similar to the gamma cells of *Sesarma*. They correspond in all features to the gamma cells. Small size, little cytoplasm relative to the size of the nucleus, and scarcity of secretory material are characteristic of these cells in both animals.

Of the four neurosecretory cell types described by Matsumoto (1954) for *Eriocheir japonicus,* he compares only his C cells, located in the ventral ganglion, with Enami's beta cells. However, Enami has shown that no beta neurosecretory cells occur in the ventral ganglion of *Sesarma*. Judging from the figures in their papers and the cell types observed in the crayfish, it appears possible that Matsumoto's C cells might be more comparable to Enami's alpha cells and to the crayfish Type 3 cells.

Carlisle and Passano (1953) found three types of neurosecretory cells in the x-organs of most species of crustaceans they examined. However, the number of cell types later was reduced to two (Carlisle, 1953). These authors showed that in the Natantia, the x-organ is divided into two portions, the pars ganglionaris which is located on the medulla terminalis and the pars distalis which is located elsewhere in the eyestalk. The Brachyura and the crayfish, in contrast to the Natantia, possess an undivided x-organ. Also, Carlisle and Passano found one neurosecretory cell type to be located in the pars ganglionaris x-organi and the other in the pars distalis x-organi. The cells of the pars ganglionaris x-organi are comparable to the giant beta neurosecretory cells of *Sesarma*, and Carlisle and Passano referred to them as the *x-organ neurosecretory cells.* It is evident that, since the Type 2 neurosecretory cells of the crayfish are comparable to the giant beta neurosecretory cells of *Sesarma,* they are also similar to the *x-organ neurosecretory cells* described in the Natantia by Carlisle and Passano.

There is a close parallelism in the arrangement of neurosecretory cell groups of the crayfish and the land crab, *Gecarcinus* (Bliss, Durand and Welsh, 1954). Furthermore, a comparison of Figure 8 of the present paper with Figure 11 in Enami's paper (1951) has already revealed that there is a remarkable similarity in the distribution of neurosecretory cell types in the crayfish and *Sesarma*. The parallelism in the distribution of neurosecretory cell types in the crayfish and *Sesarma* is particularly interesting when the physiological role of these cells is considered. This is discussed in the next section.

Secretory activity

When considering the increase in secretory activity that was observed in one type of neurosecretory cell in May, it should be remembered that the animals used in May had been kept in the laboratory for three to four months. There is evidence that crustaceans kept in the laboratory for long periods of time are different from those freshly collected. The molt-promoting effects of constant darkness on *Gecarcinus* (Bliss, 1954) are slowed down or delayed when freshly collected crabs are used. Animals long maintained in the laboratory respond quickly (Bliss, personal communication). *O. virilis,* kept in the laboratory, have been observed to molt in fairly large numbers in May and the first part of June. Although no observations were made on the molting of *O. virilis* in the field in May and in early June, it seems reasonable to assume that the laboratory stock animals molt at an earlier date than animals in the field because of the higher temperatures and more regular food supply that probably exist under laboratory conditions. Therefore, data from the February and May animals used in this study may not be strictly comparable to data from crayfish that were freshly collected.

The results included in Table I raise a question concerning the physiological significance of the increased content of stainable material in the Type 2 neurosecretory cells of the x-organ just prior to molt. The idea that the sinus gland is a storage-release center for neurosecretory products (Bliss, 1951, 1953; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Passano, 1951a, 1951b, 1952, 1953) implies that there is a mechanism whereby the rate of release of the substances can be controlled. Indeed, the well known reactions of certain crustaceans to background color are evidence that the release of certain neurosecretory products, *e.g.*, chromatophorotropins, is precisely regulated. Since a molt-inhibiting substance is produced in the x-organ and passed to the sinus gland for release into the blood stream, it is necessary to assume that at some time before the animal molts there is a decreased synthesis of this substance in the cells of the x-organ, a decreased release from the sinus gland, or both. It is assumed here that the release of the molt-inhibiting substance is decreased before molt.

It is known that the pars ganglionaris x-organi of the Natantia produces a molt-inhibiting hormone (Carlisle, 1954) and is comparable to a portion of the x-organ in the crayfish (this paper). Since the crayfish x-organ probably produces a molt-inhibiting hormone and since the only neurosecretory cells present in the pars ganglionaris x-organi of the Natantia are comparable to the Type 2 neurosecretory cells of the crayfish, it is conceivable that this neurosecretory cell type is the source of the molt-inhibiting hormone. If this is so, then the accumulation of stainable material found in the Type 2 neurosecretory cells just before molt can be considered evidence of more (1) precursor of the molt-inhibitor substance, (2) carrier substance, or (3) active material.

It is evident from the cell counts of the May animals that an assumed reduction in the rate of release must occur over a rather long period before molt. In adult crayfish large amounts of secretory material are present in these cells early in May, and signs of this increase are found in February in laboratory crayfish. Preliminary studies show that in immature crayfish possessing an intermolt period of approximately thirty-five days, increased amounts of secretory material are present in Type 2 neurosecretory cells of the x-organ at least five days before molt.

Fewer Type 2 neurosecretory cells contain secretory material after molt. This could result, if, after molt, there is a sudden release of stored material from the sinus gland and a rapid transfer of material from the cell bodies to the sinus gland for further release. Pyle (1943) found pronounced changes after molt in both the amount and staining qualities of the sinus gland material. He fixed eyestalks from *O. virilis* a few hours before molt and after the animals had completed molt. He found that there was a sharp reduction in the number of secretory granules present in the sinus glands after molt. Since the secretory masses he refers to in his photographs are identical in appearance with similar masses observed by the present author in neurosecretory fiber endings in the sinus glands, it is possible that practically all of the material in a given axon ending is released after molt. In the crayfish this release takes place in a period of not more than a few hours (Pyle, 1943).

The accumulation of stainable material in the Type 2 neurosecretory cells of the x-organ prior to molt has been explained on the basis of a hypothetical witholding of molt-inhibiting hormone by the sinus gland and a continued synthesis of hormone or its precursor in Type 2 neurosecretory cell bodies of the x-organ. The secretory activity of the Type 2 neurosecretory cells is summarized in Figure 11. This is in complete agreement with the existing hypothesis on the control of molt in crustaceans (Bliss, 1953; Bliss, Durand and Welsh, 1954; Bliss and Welsh, 1952; Passano, 1953). It is interesting that the only neurosecretory cells of the eyestalk that show histological changes correlated with molt are restricted to the x-organ, the only cell group so far proved to be effective in the prevention of molt (Passano, 1953). As to the functions of the other neurosecretory cell types in the crayfish, no information was obtained in this study.

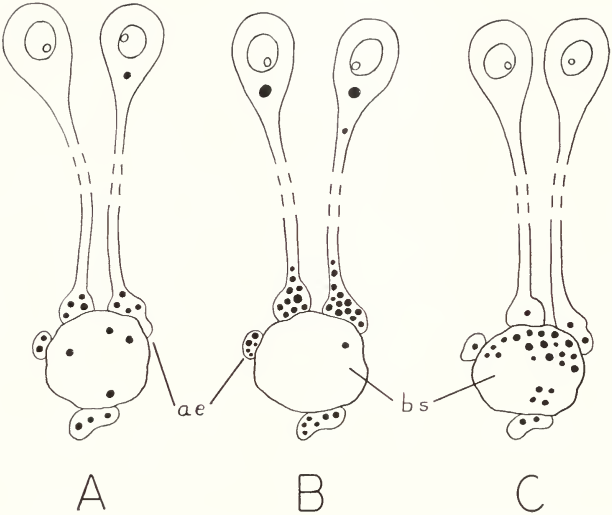

FIGURE 11. Hypothetical scheme for the secretory activity of Type 2 neurosecretory cells. A. During the intermolt period, a slow release of secretory material into the blood and synthesis of the material in Type 2 neurosecretory cell bodies continues. B. Shortly before molt, release of neurosecretory material into the blood is decreased; synthesis of the material in the Type 2 neurosecretory cell bodies continues. Material thus accumulates in the axon endings and in the cell bodies. C. Immediately after molt, a sudden release of secretory material into the blood occurs; cell body secretory material is transferred quickly to the axon endings for release. ae, axon ending; bs, blood sinus.

SUMMARY

1. There are four cytologically distinct types of neurosecretory cells in the eyestalk and brain of *Orconectes virilis.* Two of these neurosecretory cell types are restricted in their distribution to the x-organ. The other two cell types occur in all neurosecretory cell groups in the eyestalk and brain except the x-organ.

2. The distribution of neurosecretory cell types has been compared with that described by Enami (1951) for *Sesarma.*

3. The Type 2 neurosecretory cells are the only neurosecretory cells that undergo histologically demonstrable changes in secretory activity in relation to the molting cycle. It is suggested, therefore, that the Type 2 neurosecretory cells are the source of the molt-inhibiting hormone.

4. Arguments are presented in favor of the view that at some time before molt a decrease occurs in the rate of release of molt-inhibiting hormone from the axon endings of the Type 2 neurosecretory cells. This decrease seems to be correlated with a concurrent accumulation of stainable material observed in Type 2 neurosecretory cell bodies.

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THE PRESENCE AND SIGNIFICANCE OF RESPIRATORY METABOLISM IN STREAK-FORMING CHICK BLASTODERMS[1]

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The value of simple sugars, particularly glucose, in early chick development is well known (Needham and Nowinski, 1937; Spratt, 1949; Taylor and Schechtman, 1949; Fraser, 1954a). Needham (1931) and Romanoff and Romanoff (1949) list glucose as a free constituent of egg yolk, and recent investigation (Fraser, unpublished results) has shown that glucose is the only free monosaccharide in egg white dialysate detectable by chromatographic procedure. In 1938 Jacobson found that there was a marked glycolysis in involuting mesodermal cells at gastrulation. Such studies have illustrated the importance of carbohydrate metabolism in early chick embryogenesis.

While the importance of carbohydrate utilization is generally recognized, the manner in which it is metabolized has been disputed. Novikoff, Potter and LePage (1948) stand against the contention of Needham and Nowinski (1937) that there is a non-phosphorylating glycolytic scheme in young chick blastoderms. The former authors were able to detect assorted phosphorylated carbohydrate components in embryos of three to ten days incubation. Needham and Nowinski were unable to find an increase in oxygen consumption either in whole embryos or homogenate, when phosphorylated sugars were added.

Aside from the energetics involved at this level of glycolysis, terminal oxidation with molecular oxygen by cytochrome oxidase has been followed. Using manometric means, Potter and DuBois (1942) found the first evidence of this enzyme in the six-day embryo. Albaum and Worley (1942) were able to detect activity, as measured by oxygen uptake, in the embryo of four days. By soaking blastoderms in solutions containing dimethyl-p-phenylenediamine and alpha naphthol (nadi reagent), followed by visual inspection, Moog (1943) has been able to show that cytochrome oxidase is present even in head process stages. Sodium azide-treated embryos lost much of the respiratory activity seen in the experimental group.

Moog also found that this enzyme activity was expressed in a morphological pattern similar to that displayed by reducing enzymes (Spratt, 1951a), sensitivity to respiratory poisons (Hyman, 1927; Spratt, 1950b), anaerobiosis (Spratt, 1950a) and starvation (Spratt, 1951b; Fraser, 1954a). In general, these experiments have revealed that the node and fore-brain are regions of high metabolic activity. I (Fraser, 1954a) have been able to demonstrate that the node is very susceptible to degenerative changes on starvation, while the brain is relatively refractory to such treatment.

It is the purpose of the present paper to determine if there is indophenol oxidase

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(cytochrome oxidase according to Keilen and Hartree, 1938) activity in earlier, streak-forming stages of the chick, and if so, if there is some pattern in its distribution. Further, if certain cells do show a greater respiratory metabolism, another objective in mind is to investigate the possibility that it has some significance in the differentiation of these cells.

The results from the present work permit the following statements. Cytochrome oxidase is detectable in streak-forming chick blastoderms, particularly in the newly involuted mesoderm cells. There is no change in the activity of this enzyme in embryos on the nadi reagent following pretreatment with cytochrome *c* or albumen, but it increases appreciably in blastoderms starved for five hours in saline. There is a striking decrease in its activity, as measured by indophenol formation, in explants treated mildly with hydroquinone, a reductant presumably for cytochrome *c*. The augmented respiratory metabolism seen in newly formed mesoderm cells is related in some manner with differentiative ability at the trunk level of the chick blastoderm, since fragments of involuted mesoderm expressed ability to form mesoderm tissue, while potential mesoderm fragments (axial epiblast in broad- and intermediate-streak embryos) failed in this respect.

Experimental Procedures and Results

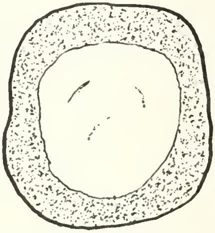
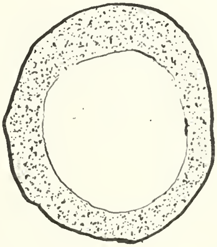
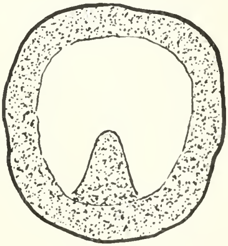
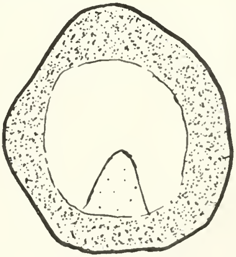
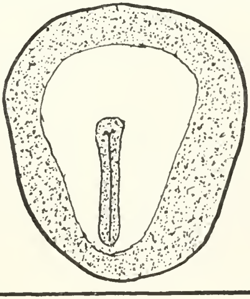
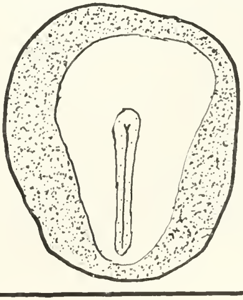
Newly laid fertile eggs were obtained from the poultry farm of the University of Tennessee. Most of the eggs used were from White Leghorn chickens, although a few were from Rhode Island Red stock. They were stored in the refrigerator at 18° C. upon receipt until the time (within five days) they were used in the experiments. The procedure for removing blastoderms from the yolk and for explanting them in culture has been given previously by Spratt (1947). Eggs were incubated in a forced draft incubator at 37.8° C., while explants were cultured at 37.6° C. The temperature in both incubators was maintained at a constant value by mercury type thermoregulators. Manipulation of the blastoderms was carried out under Ringer's solution. Specimens for histological inspection were fixed in Bouin's fluid and stained with Delafield's hematoxylin. Over six hundred blastoderms were used in the course of this investigation.

A. Aerobic enzyme pattern in early-streak blastoderms

Pre-, intermediate- and definitive-streak (DPS) embryos corresponding to stages 1, 2–3 and 4 of Hamburger and Hamilton (1951), respectively, were removed from the yolks under saline, and explanted onto freshly prepared nadi reagent, made in the following manner. Five ml. of 0.08 *M* dimethyl-p-phenylenediamine in chick Ringer's, 5 ml. of 0.08 *M* alpha naphthol in 20% ethyl alcohol-Ringer's, 1 ml. of bicarbonate buffer and 2 ml. of phosphate buffer were added to 27 ml. of Ringer's containing 300 mg. agar, after the saline-agar had been heated and then cooled to approximately 50° C. In every experiment outlined here and subsequently in which the nadi reagent was used, fresh preparations of dimethyl-p-phenylenediamine and alpha naphthol were made up immediately before use, because the former compound is oxidized rather rapidly by atmospheric oxygen. Preparation of the buffers used has been described previously (Fraser, 1954a). At this time, however, saturation of the bicarbonate buffer with CO_2 was achieved by passing the gas from a tank through a nozzle into the solution. The final pH of the 0.01 *M* nadi reagent

Table I

Pattern of cytochrome oxidase activity in early chick embryos explanted onto media containing dimethyl-p-phenylenediamine and alpha naphthol (nadi reagent) and nadi reagent with sodium azide

NO.	GENERALIZED RESULTS	
	NADI REAGENT (.01 M)	NADI REAGENT (.01 M) Na AZIDE (.005 M)
36		
34		
38		

medium was 7.1. The preparation was poured immediately into watch glasses held in petri dishes by moisture-saturated cotton rings. Gelation of the medium occurred within a few minutes. A control medium containing sodium azide at a final concentration of 0.005 M was made up in a similar manner.

The embryos were explanted onto the nadi reagent or nadi reagent-azide media and incubated for fifteen minutes at 37.6° C. At precisely this time they were removed from the media by pipette and examined under saline against a white background through a dissecting microscope.

The generalized observations are shown in Table I. Cells in the opaque area peripherad to the germ wall in all three stages tested showed a dark blue-purple coloration on the nadi reagent. No other pattern of coloration indicative of cellular respiration could be found in pre-streak embryos. A dark color was apparent, however, in the streak-forming region of the intermediate-streak blastoderm and along the streak and node area in the DPS explants. Other pellucid area tissues were very faintly stained. On the azide-bearing medium, the yolk-laden cells in the area opaca retained, in large part, a deep coloration, while streak tissues were essentially colorless. In fact, it was extremely difficult to see the developing or full streak against the white background in embryos removed from the medium containing the azide.

The logical interpretation to be made from the above results is that the coloration expressed in the vitelline cells surrounding the embryo is due mainly to a non-enzymatic mechanism. Since interest was directed toward a localization of activity in the embryo proper, this issue was not pressed, although it may be conjectured that the amount of tissue and yolk in this region may be sufficient to soak up enough indophenol from the surrounding fluid to give this appearance. Other interpretations may be advanced, but failure of the azide to prevent coloration must mean that known oxidative enzymes are not involved. Furthermore, the marked depression in indophenol formation in streak tissues on the azide medium must indicate that this coloration is mediated by enzyme action. There is good evidence that azide inhibits the action of indophenol oxidase (cytochrome oxidase) which is directly responsible for the formation of the bluish-purple indophenol (Keilin, 1936) as well as transphosphorylation and ATPase activity (Meyerhoff, 1945).

To insure that the darker color in the region of the forming and full streak was not due simply to the presence of more cells compacted at this region, fragments of tissue of comparable thickness were removed from streak epiblast, mesoderm and hypoblast for inspection. For comparison, fragments of non-axial epiblast were also examined. These pieces were placed side by side on a microscope slide in a small amount of fluid and covered with a cover slip. The stained tissues prepared in this manner were examined for intracellular indophenol deposition.

Figures 2 and 3 will reveal that enzymatic activity is greater in involuted mesodermal cells than in overlying epiblast (potential mesoderm in intermediate-streak embryos) cells. Attention is drawn to the fact that indophenol is produced at the surface of small droplets in the cells. The cytoplasm of the cells is relatively free from coloration. This is typical of all cells observed. It may also be seen that while the number of droplets is essentially the same in both epiblast and mesoderm cells, the enzymatic activity is greater on the surface of those in newly involuted cells. These globules are readily stained with Sudan III, indicative of a lipid content. These photographs are of living cells removed from a streak-forming blastoderm. In obtaining the photographs care was taken to make sure that identical conditions, such as illumination, exposure time, time of processing, etc., were maintained. The similar appearance in the photographs of a defect in the lens of the photographic equipment will attest this. Such inspection revealed that as far as indophenol oxidase activity is concerned, streak hypoblast and all epiblast cells tested were the same. It is clear that the darker coloration of the region of the forming streak or in the node and full streak of the older blastoderms is due solely to a greater respiratory activity in newly involuted mesoderm cells. This observation is in conformity with that of glycogen utilization by invaginating mesoderm made by Jacobson (1938).

B. *Modification of cytochrome oxidase activity by pretreatment*

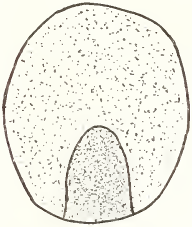
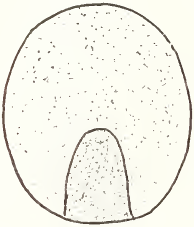
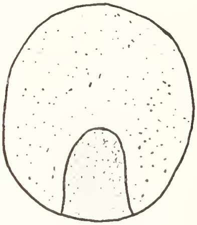
We are dealing here with an enzyme which has as its substrate the nadi reagent under experimental conditions and presumably cytochrome *c* in normal cellular respiration. Therefore, on theoretical grounds at least, it should be possible to modify the reaction between the enzyme and the nadi reagent by the addition of the normal substrate. If living cells behave as does mammalian heart muscle extract, according to the observations of Keilen and Hartree (1938), we should expect an increase in oxidation of the diamine on addition of cytochrome. At the same time, a depletion of readily metabolizable food reserves in the cell, resulting in a depressed enzyme activity, could also conceivably lead to greater nadi oxidation, and hence augmented coloration. These ideas were followed by the following experimentation. Four dozen eggs were supported on their sides in a tray and left in the refrigerator at 18° C. overnight, so that the position of the blastoderm would be known. On the following day 0.2 ml. of 1.4×10^{-4} *M* cytochrome *c* was injected into the yolk sacs of two dozen eggs, while a similar quantity of 2% sodium succinate was injected into the other two dozen. Based on previous measurements of frozen eggs a needle of sufficient length was chosen so that the injected materials would be placed about one quarter inch from the blastoderm. The needle was inserted vertically from the lower side of the egg to avoid possible injury to the blastoderm. Both preparations that were injected were sterilized by filtration. The concentration of the cytochrome *c*, prepared according to Umbreit *et al.* (1949), was established by use of the Beckman spectrophotometer. The eggs thus treated were incubated for ten hours after which they were explanted onto the Nadi reagent in the manner described above.

The results were rather disappointing in that there was no difference in enzymatic activity in either of these groups of embryos as compared to normal, non-injected controls. It became clear that the question as to whether the negative results were due to the inactivity of the materials on the blastoderms or to the failure of the materials to reach the embryos could not be resolved by this procedure, so it was abandoned.

Next, early streak blastoderms were removed from the yolk in the usual manner after ten hours of incubation, and incubated in various liquid preparations. These included: (1) saline, (2) albumen, (3) albumen-cytochrome *c*, (4) albumen-cytochrome *c*-hydroquinone and (5) albumen-hydroquinone. The final concentration of cytochrome *c* was $1.4 \times 10^{-5} M$, that of hydroquinone $10^{-3} M$. The albumen concentration was the same as previously employed in agar gels. Pretreatment time for those in the first three media indicated was five hours. It has been shown (Spratt, 1951b; Fraser, 1954a) that permanent damage occurs in embryos explanted on non-nutrient media for intervals longer than this. Blastoderms were pretreated in the media containing hydroquinone for one hour, since beyond this time cell dispersal began. Twenty-four embryos were placed in each medium, incubated for the period indicated above, removed, washed thoroughly in saline and immediately explanted onto a nadi reagent-bearing agar medium. On this they were again incubated for fifteen minutes after which they were removed, placed in Ringer's and inspected. Embryos of the same age were removed directly from eggs and treated on the nadi reagent for the same length of time for comparison.

TABLE II

Relative expression of cytochrome oxidase activity in streak-forming chick blastoderms pretreated with materials indicated

	GENERALIZED RESULTS		
PRETREATMENT MEDIUM	SALINE	ALBUMEN ALBUMEN—CYTOCHROME C CONTROL	ALB.-CYT. C—HYDROQUINONE ALBUMEN—HYDROQUINONE
			
NUMBER	24	60	48

The results are shown in generalized form in Table II. To avoid confusion, the three groups of media producing different results will be treated separately.

(1) *Albumen, albumen-cytochrome c, control* (*no previous treatment*): the sixty blastoderms in this group were all similar to those described previously with respect to indophenol oxidase pattern and intensity of color. It appears obvious that neither the albumen nor the cytochrome *c* had any affect on the enzyme activity when presented to the embryos in this manner.

(2) *Saline* (*non-nutrient*): Blastoderms incubated in this fluid stained most intensely by nadi reagent. All embryonic tissues were slightly darker than those on media listed above, but streak tissue was considerably more colored. A comparison of cellular details in fragments of tissues from streak epiblast, mesoderm and hypoblast between these and albumen-treated embryos revealed that coloration was darker in all three germ layers in prestarved embryos, with newly involuted mesoderm again showing the greatest indophenol deposition (Fig. 4).

(3) *Albumen-cytochrome c-hydroquinone, albumen-hydroquinone:* After pretreatment in these media, blastoderms showed a striking decrease in indophenol coloration when incubated on the nadi reagent. All tissues seemed to be stained somewhat less, but again the streak-forming tissues seemed most influenced by pretreatment. Although not nearly as faintly colored as those on an azide medium, these tissues nevertheless were considerably lighter in appearance than in control blastoderms.

In all cells observed, the blue color was localized on the surface of intracellular globules, even in starved embryos. It is interesting to note that cells showing the greatest enzyme activity are those of starving blastoderm mesoderm, and that this activity is on the surface of lipid material. The significance of this and of other observations made at this time will be discussed more fully later. It will suffice to point out here that the less intense color on the droplets in cells of hydroquinone-treated animals represents a decrease in dimethyl-p-phenylenediamine-alpha naphthol oxidation. This is what one would expect if one assumed that the hydroquinone acts specifically as a reductant (as has been shown by Krahl and co-workers, 1941, in sea urchin eggs) for intracellular cytochrome *c* and not indophenol, and provided that the cells were not killed by such treatment.

Considering the first assumption, it was determined that hydroquinone, in the concentration used in the experiment, and even in much greater concentration, could neither prevent the formation of indophenol from fresh nadi reagent, nor could it reduce indophenol to the leuco form *in vitro*. Secondly, other blastoderms treated with hydroquinone as indicated were subsequently washed thoroughly and explanted onto an albumen-agar medium. These were then incubated for twenty-four hours. Although development did not proceed as in normal explants, there was some slight morphogenesis, and tissues did not have an opaque appearance characteristic of death of the cells.

C. *Non-autonomy of the increase in cytochrome oxidase activity*

The question arose as to whether the increase in enzymatic activity seen in involuted mesoderm was a function of time or of location of tissue. Preceding statements have indicated that after a pretreatment interval of five hours, there was less indophenol localized in epiblast cells than in mesodermal cells. But coincident with change in time there has been some involution during pretreatment.

Small fragments of tissue taken from streak epiblast and streak mesoderm were removed from streak-forming blastoderms and cultured under albumen (prepared as previously outlined) for intervals from five to ten hours. These were then washed in saline and explanted onto the nadi reagent medium for fifteen minutes and inspected. Similar fragments removed from blastoderms of the same age, but removed directly from eggs, were stained as controls.

The cultured streak epiblast tissue had the same blue indophenol coloration as the controls. Streak mesoderm cells from both groups also looked identical, although darker in appearance than epiblast cells.

By preventing involution in this manner in epiblast tissue (prospective mesoderm), cultured for a sufficient period of time for this basic morphogenetic phenomenon to have occurred, it was thus possible to show that increase in indophenol oxidase activity is not autonomous in this tissue. It seems clear that the gain in respiratory activity is either due to movement of cells through the streak in gastrulation or to the influence of surrounding cells in a new mesodermal location. The following experiments are directed toward this question.

D. *Significance of increased respiratory activity of cells in histogenesis*

If an increase in enzyme activity in cells is due simply to placement of the cells, it should conceivably occur in streak epiblast cells implanted in a mesodermal location in early chick embryos. Eighteen fragments of such tissue were implanted through small tears in the hypoblast into positions indicated in Figure 1. Fragments were removed from stage 2 (Hamburger and Hamilton, 1951) embryo streak epiblast, while hosts were of stages 2 and 4. Blastoderms with implanted tissue were then incubated for six hours on a regular albumen-agar medium, after which

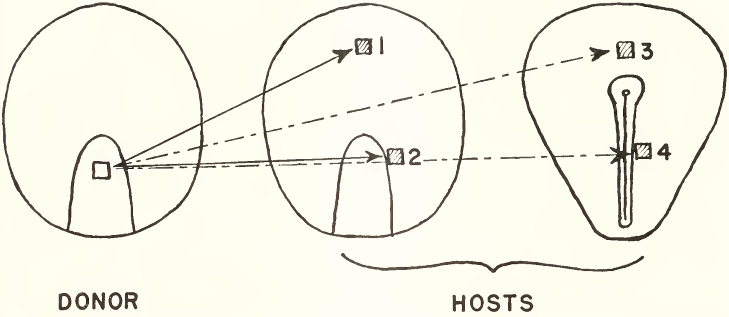


FIGURE 1. Illustration of sites for implanting fragments of streak epiblast into mesodermal locations of intermediate-streak and DPS blastoderms.

they were removed and explanted onto the nadi medium and reincubated for fifteen minutes. The implants, when found, were then removed under saline and mounted on microscope slides along with nadi-stained fragments of epiblast removed directly from streak-forming embryos for comparison. About one-third of the implants were extruded during incubation on albumen and were hence lost.

Fragments placed at mesodermal positions 2 and 4 in Figure 1 looked identical to control pieces. There had been no increase in enzyme activity in cells placed in these positions. From this and the foregoing observation, we might conclude that the increase in cytochrome oxidase activity in newly involuted cells is neither a direct function of time nor location, but is tied in with the morphogenic movement of the cells through the streak. This at least seems true for cells at the level of the streak in the early chick blastoderm.

It was rather surprising to see that implants into mesoderm in the prospective head region (positions 1 and 3 in Figure 1) were considerably darker than the controls. On the cell level, there was perceptibly more indophenol on the globules in cells of the implants than of the control fragments. At this region of the embryo, it appears that the development of intracellular catalytic activity is related to location of the cells.

These experiments were then followed by others to test the significance of this increase in observed enzymatic activity with respect to differentiative ability of the tissues involved. Fragments of streak epiblast were excised from streak-forming embryos and marked very lightly with diluted India ink under saline. They were then placed in saline and larger particles of carbon were removed with a steel needle. After a final washing, the pieces of tissue were implanted into mesodermal sites indicated in Figure 1, in both streak-forming and DPS hosts. Implantation occurred through small tears made in the hypoblast at the desired regions. In a similar manner, small pieces of newly involuted mesoderm from the same embryos were implanted in the same regions with the exception of the head region in early streak embryos. The host blastoderms were then cultured on albumen-agar for twenty-four hours at 36.7° C., fixed with Bouin's fluid and prepared for paraffin impregnation. The serial sections were made 12 microns in thickness. Eighteen implants were made into each site indicated.

Table III

Summary of results from implanting fragments of streak epiblast of streak-forming (SF) chick embryos into mesodermal sites of host embryos.

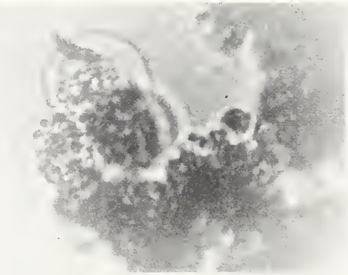
Type of fragment	Site of implantation	Fragments recovered*	Results
Epiblast	Head SF	12	Head mesoderm and pharynx
Epiblast	Head DPS	9	Head mesodern and pharynx
Epiblast	Flank SF	15	Isolated ball degenerative cells
Epiblast	Flank DPS	13	Isolated ball degenerative cells
Streak meso.	Head DPS	12	Head mesoderm and pharynx
Streak meso.	Flank SF	10	Ball of living cells Flank mesoderm
Streak meso.	Flank DPS	10	Ball of living cells Flank mesoderm

* Number of blastoderms showing some evidence that tissue had been implanted.

The results are given in Table III. It will become immediately evident that there is a good correlation between increased cytochrome oxidase activity and differentiative ability. Fragments of epiblast placed in prospective head mesoderm not only show an increase in metabolic (oxidative) activity but also display an expanded capacity for differentiation. Implants in flank regions failed to show any increase in enzymatic activity coincident with a failure to produce mesodermal structures. It is also apparent that involuted mesoderm has the capacity to form both mesoderm and endoderm in the head region as well as flank mesenchyme. There are limitations to this ability at the trunk level, however. Figures 5–10 are photographs illustrating the results obtained. Engulfed particles located in head mesoderm and pharynx are taken as evidence that these cells had differentiated from implanted tissue.

Discussion

The work of Moog (1943) has established that cytochrome oxidase is present in chick embryos in stages as early as those possessing a head process. In view of the fundamental importance of this enzyme in many diverse organisms, there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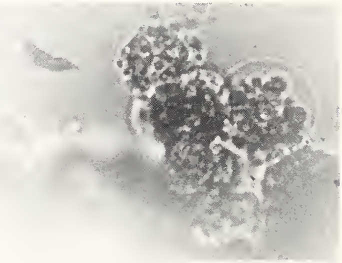
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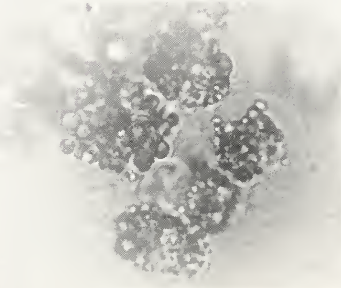
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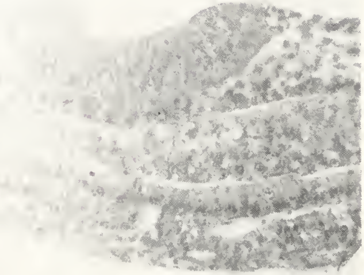
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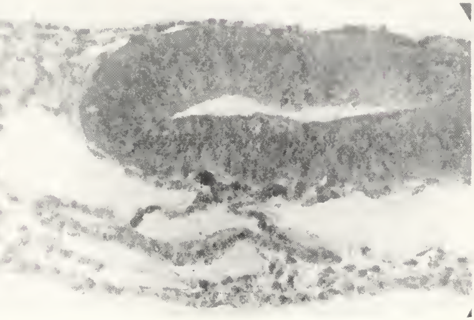
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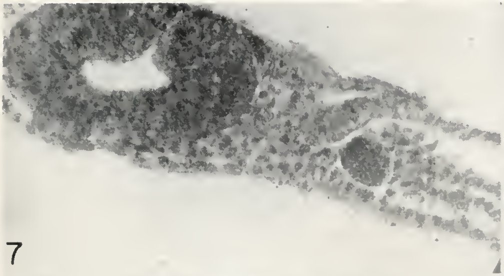
FIGURE 2. Living nadi reagent-treated cells from streak epiblast of an intermediate-streak blastoderm. Note that indophenol is deposited on the surface of intracellular droplets. × 960.

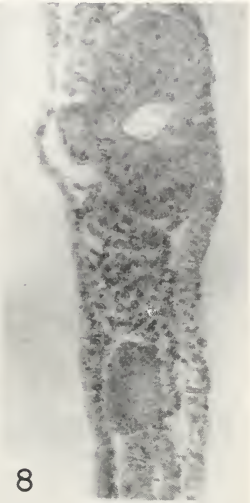
FIGURE 3. Photomicrograph of living cells stained on the nadi reagent. These cells are from newly involuted mesoderm of an intermediate-streak embryo. × 960.

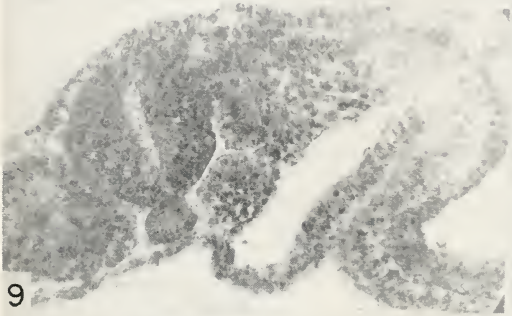
FIGURE 4. Stained streak mesoderm cells from a pre-starved intermediate-streak blastoderm. × 960.

FIGURE 5. Carbon-marked cells in pharyngeal endoderm of a stage *2* explant after twenty-four hours of subcultivation. The brain at this level has not rolled completely into a tube. × 120.

FIGURE 6. Carbon engulfed by mesoderm and pharynx of a DPS explant after one day of subcultivation. ×120.







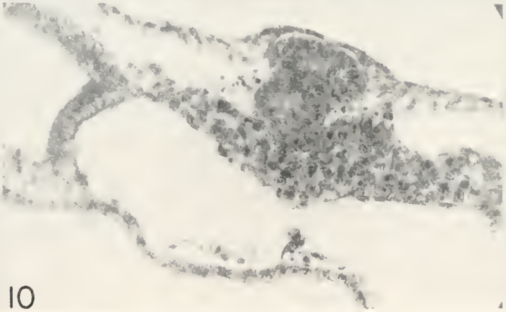


FIGURE 7. Remnant of an epiblast implant grafted into flank mesoderm of a DPS host followed by twenty-four hours of incubation. ×120.

FIGURE 8. Photomicrograph of an isolated ball of degenerative epiblast cells in flank mesoderm of a stage 2 blastoderm host after one day of subcultivation. × 120.

FIGURE 9. Isolated mass of viable cells in mesoderm initially implanted in the flank region of a DPS host. Photograph taken twenty-four hours postoperatively. Donor cells were from newly involuted mesoderm of a stage 2 blastoderm. × 120.

FIGURE 10. Photomicrograph of carbon-marked flank mesoderm cells in a stage 2 host following one day of subcultivation. Donor tissue was newly involuted mesoderm from a stage 2 embryo. × 120.

is no reason to believe that it may not be present in even earlier chick blastoderms. It has been detected by Krahl and co-workers (1941) in pre- and post-fertilized Arbacia eggs. Regardless of the type of food being utilized, as long as aerobic oxidation occurs it seems likely that this terminal enzyme will be involved. It is common knowledge that the chick embryo uses oxygen continuously following laying of the egg. The results of the present investigation have shown that cytochrome oxidase is indeed present in very early embryos, detectable by the nadi reagent under the conditions utilized even in streak-forming stages. Before this time it is probable that this enzyme is present but is not specifically outstandingly active at any localized region.

The specific significance of the observed rise in activity of cytochrome oxidase in involuting cells can only be conjectured. Certainly it is associated in some manner with the differentiative process, since it is accompanied by an expansion of possible fates in cells in which it occurs. It may well be that the increased energy liberated in these cells at this time may be directed toward anabolic processes. That differentiation is accompanied by aerobic oxidation has very recently been pointed out by Warburg (1956). The correlation between oxidative activity and histogenesis appears of prime significance.

Previously (Waddington, 1932; Fraser, 1954b) it has been shown that an interchange of cells between head endoderm (pharynx) and head mesenchyme is much in evidence in early chick embryos. It may well be that tissues in this region are more influenced in their differentiation by cells about them than they are at lower (trunk) levels. No evidence has been found in the present investigation to support the observations of Waddington and Taylor (1937) that epiblast tissue implanted at lower regions of the chick blastoderms would form mesodermal structures.

Mention should be made of the results obtained in experiments dealing with nadi oxidation in blastoderms pretreated with various materials. If we assume that there is competition between the nadi reagent and cytochrome *c* for the enzyme cytochrome oxidase, then certain interpretations of the results can fruitfully be made. This assumption is in sharp contrast to the ideas of Keilen and Hartree (1938), but see below. It is well known that both of these materials are oxidized by this enzyme in the presence of molecular oxygen to indophenol and oxidized cytochrome *c*, respectively. If we accept the assumption of a competition of substrates, the increase in nadi oxidation in starved embryos could mean that normal oxidation through cytochrome *c* is curtailed, presumably due to exhaustion of utilizable carbohydrate reserves (free hexoses). Dimethyl-p-phylenediamine-alpha naphthol would therefore be oxidized more readily, leading to the more pronounced coloration observed.

Indophenol intensity was the same in embryos pretreated with albumen-cytochrome *c* as in controls. At the same time, embryos incubated in albumen-cytochrome *c*-hydroquinone and albumen-hydroquinone were perceptibly less colored. If cytochrome *c* could enter the cells, we should expect a decrease in nadi oxidation. The fact that hydroquinone in the absence of cytochrome *c* produced the same result as with it suggests that the cytochrome is not gaining entrance to the cells. Krahl *et al.* (1941) and Keilen and Hartree (1938) using preparations of Arbacia eggs and mammalian heart muscle, respectively, have shown that there is an increase in cytochrome oxidase activity proportional to the amount of cytochrome *c* added. The size of the cytochrome (molecular weight of 13,000 according to

Potter, 1950) should not be a serious detriment to cell entry, since larger compounds are suspected of entering cells. Nevertheless, the results would indicate that it did not enter the cells. It is obvious, however, that the hydroquinone had. In view of the fact that this material will not reduce indophenol, but has been shown to reduce cytochrome *c* (Krahl *et al.,* 1941) one is led to the conclusion that the hydroquinone selectively and persistently reduces the normally present intracellular cytochrome *c*, and hence the affinity for the oxidase with the nadi reagent is reduced.

There is, however, an alternative explanation which is more in keeping with the conclusions of Keilen and Hartree (1938). These workers found that the catalytic action of cytochrome oxidase on p-phenylenediamine was greatly enhanced by the addition of cytochrome *c*, and that this aromatic amine was oxidized much more readily by the enzyme than were other compounds, including hydroquinone. They state further that the rate of catalytic hydroquinone oxidation may be increased 30- to 40-fold on the addition of cytochrome *c* (10^{-5} to 10^{-4} M) to the preparation. Thus, rather than there being a competition between cytochrome *c* and hydroquinone for the enzyme, in heart muscle preparations at least, there is a dependency on the presence of the cytochrome for the catalytic oxidation of hydroquinone. Assuming this and again considering only the hydroquinone as entering the cell, it must be that intracellularly the hydroquinone has more affinity for the enzyme than has the nadi reagent. This is, of course, somewhat at variance with the English workers' observations. It may be that the difference in results lies in the materials and methods used. Intracellularly, structure may provide results differing from those obtained *in vitro*. At any rate, this interpretation is also in keeping with observations made.

Finally, consideration should be given to the perplexing problem of starvation of the chick embryos whose cells are amply supplied with high energy food material. Reference has already been made to the fact that chick blastoderms soon die when explanted on non-nutrient media. Spratt (1951b) has shown that recovery is possible in embryos starved for six hours on a saline-agar medium, when they are returned to an albumen substrate. I have found (Fraser, 1954a) that certain degenerative features are obvious in explants starved for ten hours. In checking recently, I have found that in embryos starved for ten hours, there are still many intracellular Sudan III-stainable globules. It thus becomes evident that lipids are not utilized, at least to any appreciable degree, by early chick blastoderms. This conclusion had been drawn previously by Needham (1931). This author, using R.Q. determinations as a basis, stated that during chick embryogeny carbohydrates are utilized for the first seven days of incubation (R.Q. = 1), proteins are used next and finally lipids are used only near the time of hatching.

SUMMARY

1. Cytochrome oxidase has been detected in chick blastoderms as early as the intermediate-streak stage, by use of the explanting procedure on an agar medium containing dimethyl-p-phenylenediamine-alpha naphthol (nadi reagent). Intracellular indophenol deposition was localized on the surface of lipid droplets, particularly in newly involuted mesodermal cells. Enzymatic activity was negligible in embryos explanted on a similar medium containing sodium azide.

2. Nadi oxidation was augmented, notably in streak mesoderm of early explants after such blastoderms had been starved in saline for a period of five hours. Embryos pretreated in albumen-saline, or albumen-saline-cytochrome *c* for a similar interval showed no increase or decrease in intracellular enzymatic activity as compared to controls, when they were subsequently explanted onto the nadi-bearing medium. However, diamine oxidation in blastoderms treated in solutions containing albumen-cytochrome *c*-hydroquinone and albumen-hydroquinone was perceptibly decreased.

3. The development of the ability to oxidize the nadi reagent was not autonomous in fragments of streak epiblast (prospective mesoderm), but required normal involution at gastrulation. This was shown by pieces of this tissue implanted into trunk-level mesoderm. When implanted in a future head mesoderm location, however, such fragments did reveal an increase in enzymatic activity. When incubated in albumen-saline for intervals of time up to ten hours, small pieces of epiblast did not show an increase in nadi oxidation.

4. These results were correlated with the ability of the tissue fragments to form mesodermal and endodermal structures. Implants of epiblast placed in prospective head mesoderm of streak-forming and definitive primitive streak hosts were incorporated into head mesenchyme and pharyngeal tissue. Similar tissue when placed with other mesoderm at trunk levels failed to differentiate into mesenchyme. Newly involuted mesoderm from streak-forming blastoderms had the same fate as did epiblast fragments, when implanted in a future head mesoderm location. At the trunk level this tissue became integrated into mesoderm cells about it or formed semi-isolated balls of living tissue.

5. The significance of the observations, with respect to nutritional requirements of early chick blastoderms and the relationship between oxygen utilization and differentiation, is discussed briefly.

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THE LOCATION OF CONTACT CHEMORECEPTORS SENSITIVE TO SUCROSE SOLUTIONS IN ADULT TRICHOPTERA[1]

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Descriptions of the mouth-parts and feeding of adult Trichoptera in recent American text books and other general works on entomology are not consistent. Folsom and Wardle (1934, pp. 20, 39), Frost (1942, p. 89), Comstock, (1950, p. 555), Metcalf, Flint and Metcalf (1951, p. 229) and Ross (1948, p. 367) described the mouth-parts as "vestigial," "rudimentary," "subatrophied," or "greatly reduced." These views carried as a corollary the belief that the adults take little or no nourishment, and Brues (1946, p. 44) listed caddis-flies with the "aphagia": insects "which do not feed at all after maturity." Borror and DeLong (1955, p. 437), however, described the mouth-parts as "chewing type, with the palpi well developed but with the mandibles much reduced," and stated that "the adults feed principally on liquid foods." Swain (1948, p. 79) also stated that adult caddis-flies take liquid food, but termed the mouth-parts a "short, uncoiled proboscis."

A review of earlier accounts reveals a similar lack of agreement. Réaumur (1737, pp. 175–176) wrote that the mouth-parts of Trichoptera are for sucking and lapping, like those of Diptera. Kirby and Spence (1826, p. 464, Pl. VII, Fig. 1), on the other hand, regarded the mouth-parts as modified mandibulate. Burmeister (1832, pp. 68; 377–378) stated that the mouth-parts are intermediate between the mandibulate and haustellate types, comparing them with those of bees. Lucas (1893) made a detailed study of the mouth-parts of *Anabolia furcata* (= *laevis*). He found the mandibles to be atrophied, the labrum and maxillae reduced, and the labium developed into a sucking organ, the haustellum. Ulmer (1904) and Cummings (1913, 1914) reported a well developed haustellum to be present in every family of Trichoptera. These facts are reported in the special works on Trichoptera by Betten (1934, pp. 19–22), Ross (1944, p. 4) and Mosely and Kimmins (1953, pp. 10–11), and in the text books of Packard (1898, pp. 74–75), Weber (1933, pp. 66–68; 1954, pp. 297–298), and Imms (1948, pp. 19, 411–412).

Réaumur (1737, pp. 175–176) stated that adult Trichoptera take liquid foods, and Burmeister (1832, pp. 377–378) reaffirmed this, reporting that he found them feeding on nectar of flowers. Lucas (1893) reported finding tiny particles like pollen in the folds of the haustellum, and he therefore believed that they feed on nectar. There were other workers who made observations, often quite casual, that confirmed or contradicted these ideas. These are reviewed in the papers of Siltala (1907) and Döhler (1914). Siltala (1907) gave adult *Phryganea striata* and *Limnephilus rhombicus* only water for three or four days, then placed a flowering branch of *Spirea* near them. They flew to the flowers and fed on the nectar, thus confirming the reports of feeding by adults.

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Döhler (1914) made many observations that leave little doubt that adult Trichoptera feed. He fed them on sucrose solution to which litmus was added and followed the changes in acidity in the gut as an indication of digestion. He fed ferric lactate in water and demonstrated the absorption of iron by the gut-wall. When he gave only water to 23 individuals of *Limnephilus flavicornis,* they survived for 19–40 days. When he gave sugar-water to 19 others, they lived 45–105 days. He observed feeding closely by seizing the wings of the insects and holding them as he brought drops of sugar-water to their mouth-parts. He found, in *L. flavicornis,* such greedy acceptance of the food that some individuals ruptured the intestine through over-feeding. All these laboratory observations led Döhler to conclude that adult Trichoptera feed in nature, and he supported this by reference to Siltala's observations and those of earlier workers who reported finding caddis-flies on flowers or at sweet baits used to lure moths.

Lucas, Siltala and Döhler, thus, seem to have shown convincingly that the mouthparts of some larger adult Trichoptera are functional. The labrum is reduced, the mandibles rudimentary, the maxillae modified, the maxillary and labial palpi well developed, and the hypopharynx or labium or both developed into an extensible haustellum. These species of Trichoptera, at least, probably feed in nature on nectar and other sweet substances.

The present paper reports experiments on four species of Trichoptera from two families, and observations on two other species from two more families. The purpose of the experiments was to discover the location of the contact chemoreceptors mediating feeding responses when stimulated with sucrose solution. The results further support the belief that the mouth-parts of adult Trichoptera are functional and that the insects feed in the adult stage.

Materials and Methods

The following species of Trichoptera were studied experimentally. Identifications were made by the authors with the aid of works of Betten (1934), Milne (1934–36) and Ross (1944). Dr. H. H. Ross kindly checked the identifications, and we wish to thank him for this.

Family: Phryganeidae
    Banksiola smithi—2 males, 4 females
    Ptilostomis ocellifera—1 male, 6 females
    Phryganea sayi—4 males, 8 females
Family: Limnephilidae
    Platycentropus radiatus—8 males, 9 females

All the experimental subjects were captured when they came to lights at night. They were lightly anaesthetized with ether and mounted alive by fastening the dorsal side of the thorax and the wings to a wax block on the end of a glass rod (Fig. 1). These are relatively large (*B. smithi* about 15 mm. long; the others 20–25 mm. long), and when they were thus mounted could easily be observed. Longevity was good if the animals were fed and watered daily; even with legs and other parts removed they lived for up to 26 days.

The contact chemoreceptors were located by the methods described in detail in Frings and Frings (1949). The animals were tested daily after night fall, for they responded more actively at night, even with the necessary artificial illumination, than in day light, as Döhler (1914) also noted. Before each daily series of tests, the animals were given water to satiety. It was essential that water-satiety be maintained in the subjects, because the tests involved discrimination between water and water with sucrose added. When the animals had taken all the water they would take, water was brought to the locus being tested on an artist's brush or glass microneedle and the reaction noted under a binocular dissecting microscope. This was followed by a similar trial with 1 M sucrose solution, and the reaction again noted. These presentations were repeated a sufficient number of times to be sure that the insect responded similarly or differently to the two stimuli. Such a series of trials constituted one test. There were variable numbers of tests carried out at each testing period. At the end of each daily testing period, the insects were fed to satiety on the sucrose solution. They imbibed heavily, but did not damage themselves, as Döhler reported for the animals he tested.

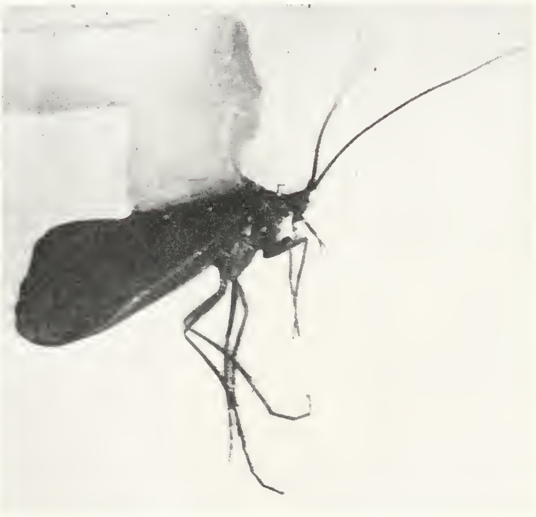

FIGURE 1. A caddis-fly (*Platycentropus radiatus*) mounted alive on a paraffin block on the end of a glass rod (3 ×).

When preliminary experiments had revealed possible loci of contact chemoreceptors, the structures were removed and the animals retested similarly. Operations were performed under light anaesthesia with paired controls anaesthetized and sham operated. For microscopic examination of possible end-organs on the experimentally determined loci, the structures were removed in 70% ethyl alcohol, transferred to 95% and thence to Diaphane on micro slides.

The following species were observed unmounted:

Family: Leptoceridae
Oecetis cinerascens
Family: Hydroptilidae
Orthotrichia americana

Attempts to mount and test about 30 individuals of the first-named were made, but these did not survive more than one day. Both of these species were very common and, as described by Döhler, highly attracted to sugar-water. They came onto the laboratory table and could be given water and sucrose solutions while they scurried about. By carefully controlling the placement of droplets near them, it was possible to test them and to observe feeding. *O. cinerascens* is large enough (about 12 mm. long) to be observed with the naked eye. *O. americana,* like all Hydroptilidae, is quite small (about 3 mm. long). It was necessary, therefore, to observe it with a dissecting microscope. Luckily the insects came right onto the brushes and needles used in testing the larger forms, and they were thus easily observed.

RESULTS

With *B. smithi* only gross localization tests were made, using brushes with water and 1 *M* sucrose solution applied to various organs of the intact, mounted animals. The palpal tips proved to be quite sensitive: touching them with sugar-water brought about extension of the haustellum. The tarsi, likewise, had contact chemoreceptors: touching them with a brush bearing sugar-water induced eager reaching with the palpi toward the brush. With water alone on a brush, in each case, withdrawal or neutral reactions were elicited. Touching the antennae with water or sugar-water resulted in withdrawal of the antennae, indicating that these lack contact chemoreceptors sensitive to sucrose.

With *Pt. ocellifera, P. sayi* and *P. radiatus* more detailed experiments were carried out: with *Pt. ocellifera* about 100 tests were made over a period of 10–26 days; with *P. sayi*, about 200 tests over 16 days; with *P. radiatus* about 300 sets of tests over 16 days. These three species reacted almost exactly alike, and the results are thus given together. Figure 2 is a photograph of the head and mouth-parts of *P. radiatus* to show the well developed haustellum and palpi.

The first series of experiments was designed to give the general locations of the contact chemoreceptors. Using brushes in paired trials with water and 1 *M* sucrose solution, no evidence of discrimination was found when the antennae were tested. If the insects were "thirsty," however, the antennae were quite sensitive to water vapor. If a brush bearing water was brought near to but not in contact with the antennae, the insects almost immediately began to reach excitedly with the palpi. Once sated with water, however, this ceased, and the only reaction to contact with a brush moistened with water or sugar-water was withdrawal of the antennae. The conclusion that the antennae lack contact chemoreceptors, however, must be stated cautiously, for recent experiments with some Lepidoptera (Frings and Frings, 1956) show that reactions mediated by the antennae may depend upon presence or absence of contact chemoreceptors on other parts of the body.

Contact of the tarsi of water-sated individuals with sugar-water elicited reaching with the maxillary and labial palpi and partial extension of the haustellum. Ordinarily the palpi were folded against the head, and this reaction was quite clear-cut. Touching only the ventral surface of the tarsus of one fore leg with the brush mediated the same response. It was impossible, however, to touch the other tarsi in an intact animal without having the fore tarsi also brought to the brush. The palpi likewise proved to be receptive: touching them with sugar-water elicited spreading of the haustellum. If 1 M NaCl solution was used instead of sucrose on the tarsi, there was no reaching with the palpi, and if it was used on the palpi, they were withdrawn sharply.

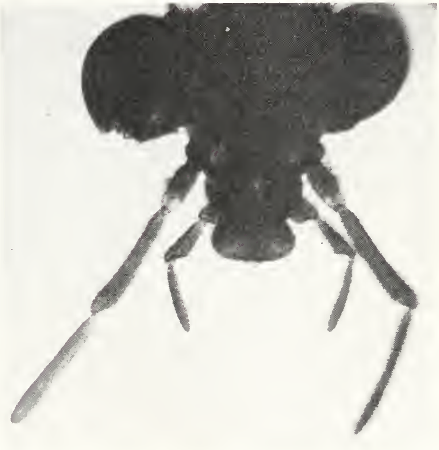

FIGURE 2. Head and mouth-parts of an adult caddis-fly (*P. radiatus*) showing the well developed maxillary and labial palpi and the medial haustellum (25 ×).

Further experiments on intact animals were made with glass micro-needles bearing water and sugar-water. The results with the antennae and tarsi were the same as when brushes were used. Touching only the tips of the maxillary palpi with sugar-water elicited reaching with both sets of palpi and partial spreading of the haustellum, much like the reaction obtained by touching the tarsi. Bringing sucrose solution to any part of a maxillary palpus other than the tip of the terminal segment brought about withdrawal, just as with water. Touching the maxillary palpal tips with NaCl solution elicited a sharp retraction of the palpi. Touching the tips of the labial palpi with sugar-water on a needle elicited spreading of the haustellum in preparation for feeding. Other parts of the labial palpi seemed not to be sensitive, as with the maxillary palpi. With NaCl solution, these palpi were also drawn away. The feeding reaction, therefore, seems to occur in two stages: 1) exploration with the palpi when an acceptable solution touches the tarsi or maxillary palpal tips, and 2) extension and spreading of the haustellum when the solution touches the tips of the labial palpi.

Following these tests, operations were performed to enable us to test parts that could not be touched without interference by known receptors. The forelegs were removed first. Using the brushes, the middle and hind tarsi together were found to be sensitive to sucrose. With care the middle tarsi together or singly could be touched, and this elicited the usual response. With the fore and hind legs removed, the middle tarsi were easily tested and found to bear contact chemoreceptors sensitive to sucrose. With the fore and middle legs removed, the hind tarsi together or singly were also found to be sensitive. All the tarsi, thus, bear the receptors. Using microneedles, the receptors were found on the ventral surfaces of the tarsi and not on the other parts of the legs, but the exact segments of the tarsi bearing them were not determined.

With the last segments of the maxillary and labial palpi removed, the reactions to contact of the palpi with sucrose solutions were abolished, thus confirming previous observations that the receptors were confined to these segments. To test the sensitivity of the haustellum, the fore and middle legs and the palpi were removed, and the animals offered water and sugar-water on brushes. A little difficulty was encountered at first, because the haustellum became covered with clotted hemolymph from the cut ends of the palpi. After this was washed off, however, the animals were able to feed. They obviously could distinguish sucrose solution from water, spreading the haustellum and drinking the former when sated with water. If NaCl was used instead of sucrose, they refused to drink. If they were drinking sugar-water and NaCl solution was suddenly substituted, they reacted by immediate withdrawal of the haustellum and often by violent retraction of the head. Thus the receptors could distinguish acceptable from unacceptable materials in solution. The receptors were not located exactly on the haustellum, but they would seem to be near the distal margins, for application to the tip of the haustellum of a droplet of sugar-water on a needle brought about almost immediate extension.

The parts of the body bearing contact chemoreceptors sensitive at least to sucrose and NaCl, therefore, are the ventral sides of all the tarsi, the tips of the terminal segments of the maxillary and labial palpi and the haustellum. Generally the reaction to appropriate stimulation of the tarsal or maxillary palpal receptors is reaching with and vibration of the palpi and partial extension of the haustellum. The reaction to appropriate stimulation of the labial palpal tips or the haustellum is extension and spreading of the haustellum and feeding. No differences were noted between the reactions of males and females.

The tarsi, palpi and haustellum of these three species were mounted on slides and examined in an attempt to find the possible end-organs involved. On the ventral surfaces of the tarsi there are many short, thin walled, trichoid sensilla among the longer hairs and spines. These are quite similar to the probable receptors in

Lepidoptera and Diptera (Eltringham, 1933; Frings and Frings, 1949; Grabowski and Dethier, 1954; Hayes and Liu, 1947; Lewis, 1954a, 1954b; Tinbergen 1939). On the palpi there are many trichoid sensilla on all the segments, but there is no way at present to select any as possible receptors. On the posterior face of the haustellum, there are basiconic and trichoid sensilla in small numbers. Either of these might be involved, because no other obvious sensilla are present, but the data do not warrant any definite selection. It is probable, therefore, that the receptors on the tarsi and palpi are trichoid sensilla, while those on the haustellum are either trichoid or basiconic.

With *O. cinerascens* only a few tests were made on mounted animals before their untimely deaths. It was obvious that they had tarsal and palpal receptors like the others tested, but these were not further localized. With this species and with *O. americana* many observations were made on unmounted individuals that visited the laboratory table where the others were being tested or came onto the brushes and needles used in the experiments. They scurried about in characteristic, excited manner, turning to and fro, antennae vibrating. If a droplet of water was in their way, they usually stopped as soon as they touched it and drank. When sated with water, they no longer stopped at these droplets. If a droplet of sucrose solution was placed in their way, however, they stopped as soon as the tarsi touched it and turned round and round reaching with the palpi. As soon as the palpi touched the droplet of sugar-water the haustellum was extended and the insect fed.

Discussion

These observations and experiments on adults of six species of Trichoptera representing four families show that, in these at least, the mouth-parts are functional, modified for sucking, and that the adults feed. These results are fully concordant with the reports of Lucas (1893), Siltala (1907) and Döhler (1914). Ulmer (1904) and Cummings (1913, 1914) reported that only a few species from one or two families lack a well developed haustellum. While all the observations on living animals have been made on representatives from only four families and mostly from two families, the conclusions may have validity for Trichoptera generally. At least no one has shown experimentally that any species does not feed in the adult state.

The presence of tarsal contact chemoreceptors also indicates affinity with the haustellate insects. Those studied to date (Hemiptera, Lepidoptera, Diptera, Hymenoptera), in contrast with the mandibulate forms, have contact chemoreceptors on the tarsi (Frings and Frings, 1949). The near certainty that the tarsal and palpal end-organs of Trichoptera are trichoid sensilla further allies them with the typical haustellate forms. Haustellate species, in general, have trichoid sensilla for trophic contact chemoreception and mandibulate forms basiconic sensilla (Frings and Frings, 1949). In the presence of contact chemoreceptors on the palpi and the absence on the antennae, the Trichoptera are more like the Diptera than the Lepidoptera (Frings and Frings, 1949, 1956). The general form of the mouth-parts in Trichoptera also is more like that of Diptera than Lepidoptera. How much weight can be attached to evidence such as this in determining relationships among larger groups of insects we do not know. Certainly, however, further comparative studies of contact chemoreception in Trichoptera, as well as other haustellate groups, would be desirable.

SUMMARY

The loci of contact chemoreceptors sensitive to sucrose and NaCl in solution and mediating feeding responses were determined experimentally in adult Trichoptera of four species from the families, Phryganeidae and Limnephilidae. The receptors are on the ventral surfaces of all the tarsi, the tips of the maxillary and labial palpi and the haustellum. The animals feed on liquids, and these receptors allow them to distinguish acceptable from non-acceptable materials in solution. Less precise observations on two other species from two other families showed a similar situation in these. The end-organs are probably trichoid sensilla. This fact, along with the presence of tarsal contact chemoreceptors, places adult Trichoptera of these species, at least, among typical haustellate insects, most nearly resembling many Diptera in locations of the receptors and feeding reactions.

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THE FIREFLY PSEUDOFLASH IN RELATION TO PHOTOGENIC CONTROL[1]

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INTRODUCTION

The normal flashes of many fireflies are short sharp bursts of light lasting about a tenth of a second, with essentially total darkness between. The problem of how this light emission is so precisely controlled has long appealed to investigators interested in biological trigger mechanisms. Two basic facts have become firmly established: that nervous activity can initiate luminescence and that oxygen is essential for light production both in the intact firefly and in cell-free extracts (for review see Buck, 1948; Harvey, 1952; McElroy and Hastings, 1955; Buck, 1955).

There are two principal theories concerning the control of normal flashing. One postulates direct nervous stimulation of the photogenic cell, and the other proposes nervous control of the oxygen supply to the cell. There has been little empirical evidence bearing on the idea that the nerve impulse acts on the photogenic cell directly. The theory of oxygen limitation, on the other hand, has been widely supported on both physiological and anatomical grounds. Actually, as pointed out previously (Buck, 1948), all the experimental findings that have been ascribed to direct oxygen control can be equally well interpreted as effects on nervous control. The anatomical evidence is more persuasive, though circumstantial. It consists of the facts (a) that tracheal end cells under the light microscope appear to have a structure which can be interpreted as valvular (Dahlgren, 1917), (b) that end cells are present in the photogenic organs of flashing types of fireflies and absent in types that produce only sustained glows, and (c) that the end cells are strategically situated on the tracheae at the points where the tracheoles enter the photogenic tissue.

The principal experimental support for the idea that the end cell functions as a valve comes from the work of Snell (1932) and Alexander (1943). They found that fireflies exposed to low oxygen concentrations developed a dull "anoxic glow" (= our "hypoxic glow"), and when suddenly re-exposed to air produced a brilliant "pseudoflash" lasting a second or more. They interpreted these events as follows: The low oxygen narcotizes the normally closed end cell valves and causes them to open, allowing the ambient gas to enter the previously anaerobic photogenic cytoplasm. Since the oxygen concentration is low, only a dull glow develops. When air is subsequently admitted the higher oxygen concentration permits a more brilliant luminescence, which, however, then quickly dies out as the valves recover and close in the higher oxygen concentration. From these hypoxic glow-pseudoflash responses it was argued that normal flashing could also be controlled by end cell limitation of oxygen. It should be noted, however, that all the experiments involved markedly unnatural conditions and hence are not necessarily relevant to normal flash control.

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Recent experiments of McElroy and associates on cell-free extracts of firefly photogenic tissue appear to bear directly on the question of oxygen limitation versus nerve stimulation. When all the components required for light emission (luciferin, luciferase, Mg^{++}, adenosine triphosphate and oxygen) are mixed together a flash of light occurs, its intensity reaching a peak in less than 0.1 second and then declining to a low sustained level within the next 10–20 seconds. Evidence too detailed to present here indicates that this *in vitro* flash involves several reactions (McElroy and Hastings, 1955; McElroy, personal communication). First, it is believed, luciferin, luciferase and ATP react to form a luciferin-adenylic acid-enzyme "active intermediate." The intermediate is then oxidized rapidly and irreversibly, with emission of light. This oxidation corresponds to the initial rise of luminescence in the flash. However, the oxyluciferin formed during luminescence undergoes a slower, reversible phosphorylation by ATP to form oxyluciferin-adenylic acid, which strongly inhibits the enzyme in the active intermediate. This inhibition accounts for the decline in light intensity after the initial peak—a decline which takes place in the presence of excess oxygen. The eventual low level plateau of luminescence thus reflects the low concentration of uninhibited enzyme available once a steady state among the various reactions is established.

The concentration of the active intermediate—which may be considered to be the substrate of the light-producing reaction—can be changed in two significantly different ways: (1) If oxygen concentration is greatly decreased the rate of the oxidative reaction is decreased, resulting in decreased luminescence and accumulation of active intermediate. When air is readmitted the accumulated intermediate is rapidly oxidized, resulting in a flash of light. This shows that it is possible, by changing oxygen concentration, both to limit luminescence and to cause a flash. The "oxygen flash" of the extract has a remarkable quantitative resemblance to the pseudoflash of the intact firefly, which, it will be remembered, is induced by a similar sequence of changes in oxygen concentration. This suggests that both types of flash are due to oxidation of accumulated active intermediate, and that neither of them needs be oxygen-limited during its decay phase. (2) The concentration of the active intermediate may also be increased by addition of pyrophosphate, which, by opposing the formation of the oxyluciferin-adenylic acid inhibitor, frees active enzyme. Since this reversal of enzyme inhibition is rapid, lasts only until the added pyrophosphate is used up, and does not involve any change in oxygen concentration, it provides a possible model for the mechanism which induces the normal flash.

The *in vitro* system therefore suggests that the flashing of the firefly need not normally be controlled by oxygen concentration even though light production may, under some artificial conditions, become oxygen-limited. In view of this possibility, and of the ambiguity of previous oxygen-limitation experiments on intact fireflies, it is important to re-examine the evidence purporting to demonstrate end-cell control of luminescence.

Materials and Methods

The forms investigated were adults of the lampyrid fireflies *Photuris* sp. and *Photinus pyralis* from the Baltimore-Washington area, adults of the elaterid firefly

Pyrophorus atlanticus from Florida, and larvae of *Photuris*. In the males of the first two species, as in many lampyrid fireflies, the photogenic tissue occupies the ventral surfaces of abdominal segments 6 and 7. In *Pyrophorus* we investigated the small circular organs at the posterior dorsal corners of the prothorax. In the *Photuris* larva the photogenic organs are a pair of small lateral plaques on the ventral side of abdominal segment 8. Similar organs exist in the larva and pupa of *Photinus pyralis*, and sometimes persist into the adult where they function independently of the main organs.

Different gas mixtures were prepared by passing various gases through calibrated flow meters into a mixing chamber and thence to the exposure chamber, which was a 5 cm. length of glass tubing of 6 mm. bore. For visual observation up to three specimens were accommodated in the chamber, separated by wire screen partitions. A flow rate of 300–400 mL per minute was used and a reversing stopcock between mixing and exposure chambers permitted quick shifting from one gas to another, the unused gas being vented. Light intensity was measured with a photomultiplier tube apparatus (Hastings, McElroy and Coulombre, 1953) and recorded with an oscilloscope camera or a graphic meter, the firefly being held in position against the wall of the exposure chamber with a loose cotton plug.

RESULTS

1. *Responses of intact lampyrid fireflies to varied oxygen concentration*

When adults of *Photinus* and *Photuris* were exposed to various low oxygen concentrations a dim hypoxic glow usually developed, and when such glowing fireflies were suddenly re-exposed to air a pseudoflash occurred. Figure 1 illustrates a response of this sort, the hypoxic glow being represented by BC and the pseudoflash by CDE. Figure 2 shows the pseudoflash portion in more detail. An "oxygen flash," which occurs when air is readmitted to an anaerobic cell-free extract of photogenic tissue, is reproduced in Figure 3 for comparison. About 50 records of the hypoxic glow and pseudoflash have been made and analyzed, supplemented by many hundred visual observations. These in general confirm the findings of Snell and of Alexander, but certain differences were noted. For example, if a firefly was left in the low oxygen mixture after the hypoxic glow had reached its plateau level, instead of then exposing it to air, the intensity of the glow usually decreased over the course of several minutes (Fig. 1, dotted line, FG). It was also observed that some individuals failed to give a pseudoflash, or both hypoxic glow and pseudoflash, and that there was considerable variation in the length of the period between hypoxic exposure and beginning of the hypoxic glow (Fig. 1, AB), and in the intensities of both glow and pseudoflash. Also, as in nature, normal individuals sometimes showed an initial constant dim glow in air which made it difficult to recognize the start of the hypoxic glow.

In spite of these variations in response, a number of quantitative relations were apparent. First, in fireflies which had been exposed to a particular low oxygen gas mixture, the peak intensity of the pseudoflash in air was approximately proportional to the intensity of the hypoxic glow in the low oxygen mixture just prior to admission of air. Before the beginning of the hypoxic glow no pseudoflash could be elicited; during the dimmer periods of the hypoxic glow, either before or after the maximum, pseudoflashes of low intensity occurred; and during the brighter periods of the hypoxic glow more brilliant pseudoflashes occurred. In 35 experiments in which the hypoxic glow was induced with 0.25% oxygen, the ratio of pseudoflash intensity to hypoxic glow intensity varied only from 30 to 120, and the range for repeated measurements on single individuals was even smaller. Thus both pseudoflash and hypoxic glow intensities pass through a maximum with time.

A second finding was that the ratio of pseudoflash intensity to hypoxic glow intensity varied with the oxygen concentration used to induce the hypoxic glow. For example, in one typical individual the pseudoflash intensity in air was 1.5 times as great as the hypoxic glow elicited with 2.4% oxygen;[2] when 1% oxygen was used the pseudoflash was 10 times as bright as the glow; when 0.25% oxygen was used the pseudoflash was 60 times as bright; and when 0.05% oxygen was used the pseudoflash was 5000 times as bright. The increase in this ratio with progressively lower oxygen concentrations was evidently due both to diminution in hypoxic glow intensity and to an increase in the absolute pseudoflash intensity.

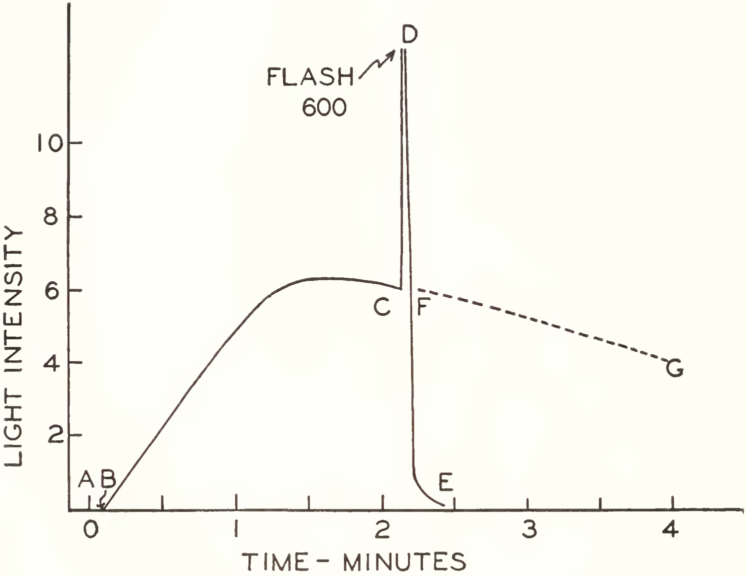

FIGURE 1. Hypoxic glow-pseudoflash response of intact male of *Photinus pyralis*, diagrammed from graphic meter record. Ordinate, light intensity in arbitrary units. At A (zero time) ¼% oxygen was introduced. At B the hypoxic glow began, AB representing the latent period. At C air was flushed through the chamber and a pseudoflash of 600 units relative intensity occurred. Had the firefly been left in ¼% oxygen at C, the hypoxic glow would have continued (FG), slowly diminishing in intensity.

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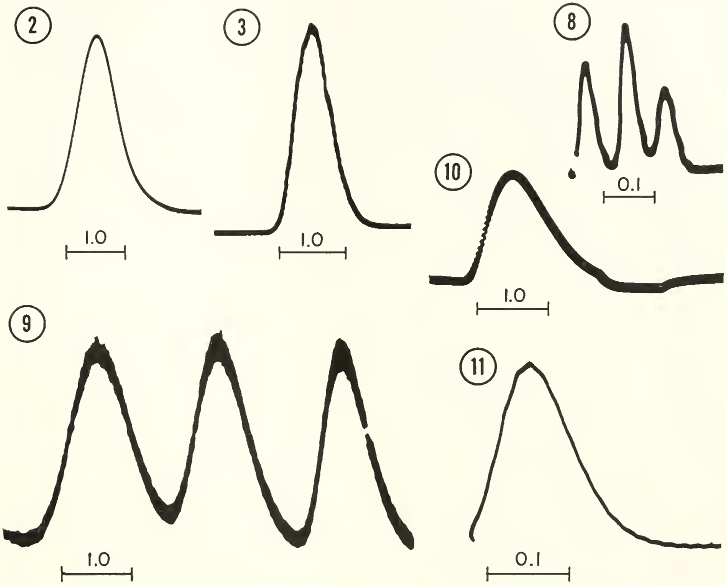
[2] No systematic attempt was made to find the upper oxygen concentration limit for pseudoflash occurrence, but it is certainly higher than the limit set by Snell (about ½%).

A third characteristic of the hypoxic glow-pseudoflash response is that the pseudoflash was remarkably constant in duration and in form (rates of accretion and decay of intensity), regardless of variations in both degree and duration of hypoxia prior to readmission of air. This was true both in repeated measurements with one individual and in records from different individuals. Intensity variations of well over a thousand-fold occurred without difference in duration. If the pseudoflash were being controlled by some sort of oxygen-sensitive effector, such as Snell supposed the end cell to be, it would be remarkable that this degree of constancy of response could be achieved, particularly in view of the individual variability in intensity and latency of hypoxic glow, and in intensity of pseudoflash.



FIGURES 2, 3, 8, 9, 10, 11. Various luminous responses photographed from the oscilloscope screen. Ordinate, light intensity; abscissa, time, with sweep going from left to right. Further descriptions in text. Time scale in seconds. FIG. 2. Pseudoflash of intact adult male of *Photinus pyralis*. FIG. 3. Oxygen flash of cell-free extract of *P. pyralis*. FIG. 8. Spontaneous flashing of intact female of *Photuris*. FIG. 9. Spontaneous glow of intact *Photuris* larva. FIG. 10. Pseudoflash of *Photuris* larva. Temporary depression in trace following flash represents period during which photocell power supply was switched off. FIG. 11. Spontaneous flash of intact male of *P. pyralis*.

2. *Oxygen responses in relation to structure*

It was shown previously (Buck, 1948) that there is no correlation between the state of spiracular valves and the times of occurrence or characteristics of the normal flash of *Photinus pyralis,* and that when intact fireflies are tested with progressively falling oxygen concentration the spiracles open well before the hypoxic glow begins and close after it ceases. Evidence that the spiracles have no immediate influence on the hypoxic glow-pseudoflash response was obtained in the present study by testing adults of *Photuris* and *Photinus* in which the spiracles of the luminous segments had been made inoperative by cautery with an electrically heated needle or by insertion of a short length of human baby hair. Although these specimens often showed a continuous glow in air, presumably caused by the mechanical disturbance, they gave pseudoflashes similar to those in individuals with normal spiracles. Likewise, it was observed that spiracular opening is regularly induced by exposure to 5% oxygen, whereas the hypoxic glow usually requires that the ambient oxygen concentration be reduced to the order of 1% to 2%. Absence of spiracular or indeed any sort of valvular influence is also seen in dead fireflies which, if prevented from drying out, may exhibit a constant dim air glow for a day or more after all visible signs of life have disappeared. Such dead specimens have permanently open spiracles yet give a pseudoflash response.

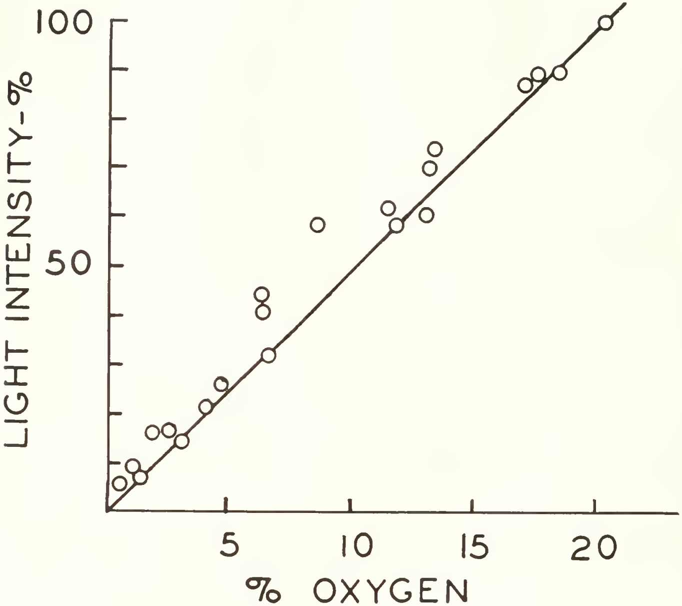


Figure 4. Relation between oxygen concentration and glow intensity (in per cent of intensity in air) of smeared photogenic tissue of males of *P. pyralis.* Data from several experiments.

A series of experiments was performed in which increasing degrees of interference with possible central nervous or tracheal control of luminescence were achieved by (a) decapitation, (b) cutting off the abdomen at the junction of the fifth and sixth segments, (c) excising the photogenic organ alone, and (d) smearing the photogenic tissue on glass. None of these preparations produced normal flashes, or indeed any continued spontaneous luminescence, except for the smeared organs, which exhibited a continuous dim glow in air, decreasing in intensity very gradually (30 minutes or more to extinction). The first three types of preparations responded to changes in ambient oxygen concentration just like intact fireflies, *i.e.*, in low oxygen concentrations they developed hypoxic glows and when air was readmitted they produced typical pseudoflashes. It is thus clear that nerve impulses originating in the central nervous system play no role in the photogenic response to hypoxia. It is also apparent that none of the tracheae external to the light organ is involved.

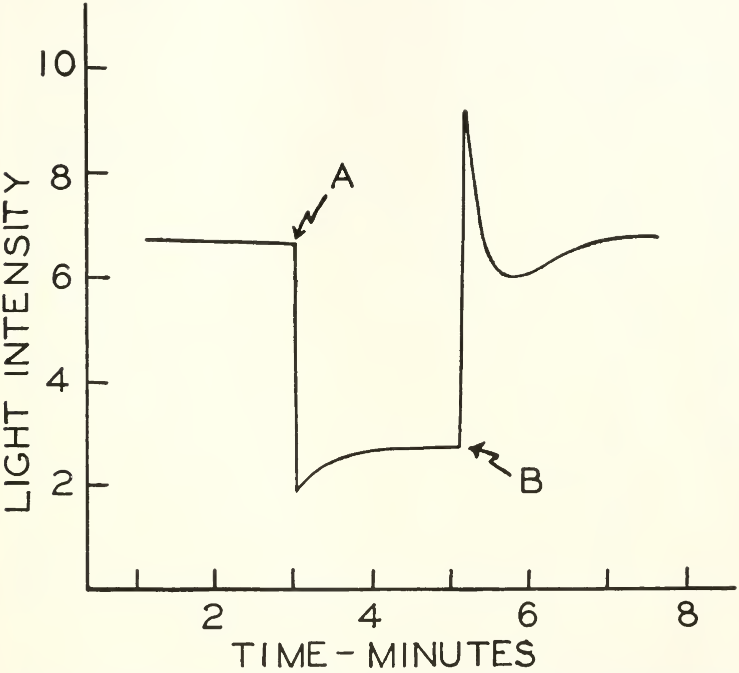


FIGURE 5. Changes in light intensity of smeared photogenic tissue of male of *P. pyralis* in varied oxygen concentrations. Tissue in air for first three minutes. At A, 8.3% oxygen was introduced. At B, air was readmitted, inducing a pseudoflash-like excess luminescence.

The intensity of the glow of the smeared organ is proportional to ambient oxygen concentration below 21% (Fig. 4) and also increases greatly in pure oxygen. Figure 5 shows the time course of the luminescence when a smeared organ was exposed (at A) to 8.3% oxygen and then re-exposed (at B) to air. The changes in luminescence are qualitatively very similar to those which occur when the same procedure is carried out with cell-free extracts (Hastings, McElroy and Coulombre, 1953). The smeared preparation differs from both the extracts and the intact organ in that its pseudoflash has a longer duration and is not so bright relative to the intensity of the hypoxic glow. These differences in glow and flash are to be expected if oxygen has become limiting in the luminescent reaction, and presumably it is the disruption of the tracheal supply within the photogenic tissue itself which is responsible for this oxygen-limitation. Similar "slow" flash responses have been demonstrated in extracts under conditions of oxygen limitation (McElroy and Hastings, unpublished). On the basis of the biochemical reactions already discussed, the intensity of the pseudoflash of the smeared organ is presumably a measure of the amount of active intermediate which has accumulated. The de-

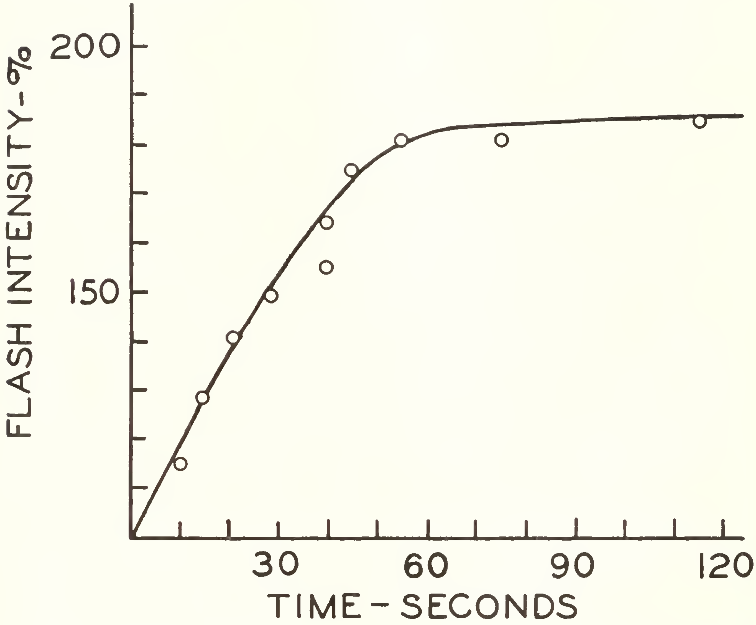


FIGURE 6. Relation between duration of hypoxia and intensity of pseudoflash in air of smeared photogenic tissue of *P. pyralis*. Each point represents pseudoflash intensity, in per cent of glow intensity in air, in an experiment similar to that diagrammed in Figure 5. The hypoxic mixture used contained 1% oxygen.

pendence of this accumulation upon both time of hypoxia and oxygen concentration in the hypoxic gas mixture is illustrated in Figures 6 and 7.

In normal males of *Photinus* and *Photuris* all the photogenic tissue in both luminous segments ordinarily participates in each flash, and apparently simultaneously.

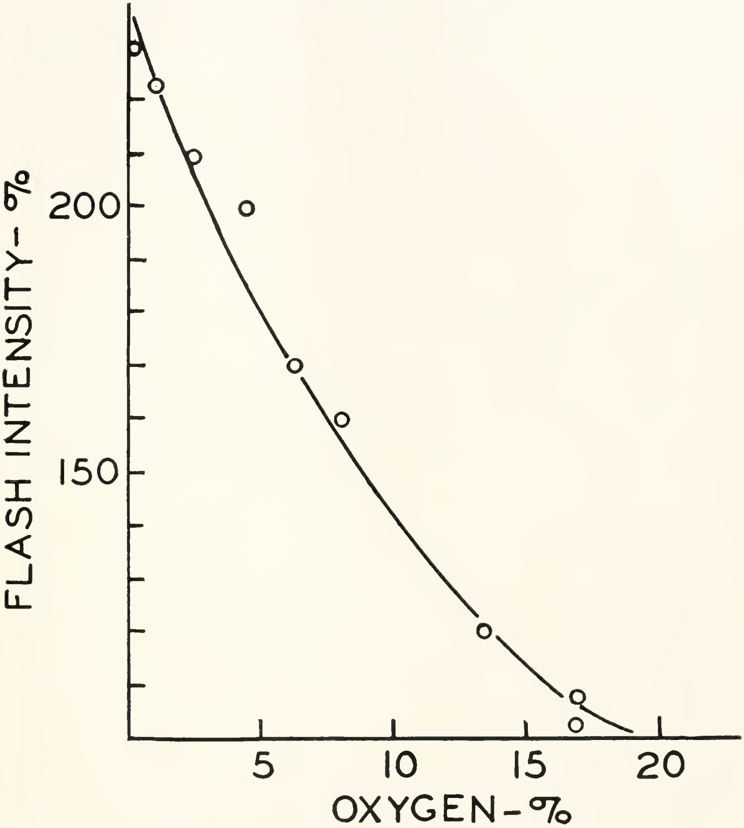


FIGURE 7. Relation between intensity of pseudoflash in air of smeared photogenic tissue of *P. pyralis,* and degree of hypoxia. Tissue exposed to each oxygen concentration for two minutes, then flushed with air. Intensity plotted on ordinate in per cent of glow intensity in air.

In some instances, however, it was observed that both spontaneous flashes and various types of induced luminescence involved only one of the segments, or only parts of one or both. Furthermore, the type of luminescence displayed sometimes differed in different regions of a single organ or even changed in the course of an experiment.

The flash of the female of *Photuris* is usually too sharp and brilliant for reliable visual observation of heterogeneity, but may possibly also involve asynchronous luminescence (Fig. 8).

In instances in which only a portion of a photogenic organ gave the hypoxic glow-pseudoflash response, the portion which failed to respond often developed a dull glow in air after the pseudoflash in the other portion had ceased. Since the photocell integrates all the light emitted, one needs to be aware of the possibility that intensity × time recordings of luminescence (*e.g.*, Figs. 1, 2) may be the resultant of two quite different sorts of things, namely, change in light intensity per unit organ area, and change in area active. We cannot exclude the possibility that an occasional heterogeneous response of this type was recorded in our work, but do not believe that any of our present interpretations is in error because of such an accident.

3. *Oxygen effects on induced glows*

Bright steady glows can be induced in both intact fireflies and isolated abdomens by air passed through cotton soaked in ethyl ether or over potassium cyanide crystals (*i.e.*, without change in ambient oxygen concentration). The dosage must be chosen to avoid either premature recovery of the animal or rapid destruction of the photochemical system (Buck, 1948). When fireflies which were glowing from exposure to ether or cyanide vapor were exposed to low oxygen concentrations, luminescence abruptly declined to a low level, then rose somewhat as an hypoxic glow. When air was readmitted a typical pseudoflash occurred. This illustrates the occurrence of a flash under conditions in which tracheal end cells would be expected to be inactivated.

4. *Oxygen responses of Pyrophorus, Photuris larva and persistent pupal organ of Photinus pyralis*

The photogenic organs of the large elaterid firefly *Pyrophorus* and of the larvae and pupae of lampyrid fireflies offer an interesting contrast to the organs of adult lampyrid fireflies in two respects. First, they never normally flash, but emit light in long-sustained glows at irregular intervals. Second, they lack the tracheal end cells which are characteristic of the flashing-type adult lampyrid organ. The pupal organs frequently persist into the adult, thus combining both organ types in the same individual.

Observation of oxygen effects in *Pyrophorus* is complicated by the fact that the glow normally fluctuates cyclically in intensity, at frequencies varying from about one peak per second to one per five seconds or slower, as observed also in a Cuban species by Harvey (1931). In addition, the intensity of the glow increases markedly when the creature is disturbed. Thus hypoxia sometimes proves sufficiently irritating that the light emitted is at first actually brighter than in air, and there is no initial decline due to oxygen limitation as in adult lampyrids glowing in air. The luminescence is only oxygen-limited at ambient concentrations of 1% or lower. *Pyrophorus* in low oxygen concentrations responds to a sudden increase in oxygen by emitting a pseudoflash which is qualitatively similar to the typical adult lampyrid pseudoflash, but often appears after a quite long latent period (up to 20 seconds) and lasts much longer.

The minute photogenic organs of the larva of *Photuris* and the persistent pupal organs of *Photinus pyralis* emit a fluctuating luminescence strikingly similar to that of *Pyrophorus* (Fig. 9). Their responses to oxygen have not been followed in detail except to confirm Buck's observation (1946, 1948) that low ambient oxygen induces an hypoxic glow and subsequently raised oxygen elicits a pseudoflash. A record reproduced in Figure 10 shows that the larval pseudoflash closely resembles that of the adult. The persistent pupal organ in the adult usually (but not always) gives an hypoxic glow-pseudoflash response in parallel with that of the adult organ.

Discussion

Evidence presented above has shown that neither spiracle, main trachea nor central nervous system is necessary for either the appearance or disappearance of luminescence in the usual type of hypoxic glow-pseudoflash response. The possibility that tracheal end cell valves might be involved in the response is likewise all but eliminated by the following considerations: (a) Pseudoflashes occur in *Pyrophorus*, the *Photuris* larva and the persistent pupal organ of *Photinus pyralis* (all of which lack end cells), and in lampyrid fireflies treated with cyanide and ether (where end cell valves should be inactivated); (b) as already pointed out, the constancy of pseudoflash duration makes it difficult to believe that an end cell mechanism is functioning in the control; (c) recent electron microscopy by Beams and Anderson (1955) casts grave doubt on there being any valvular structure in the end cell. In fact the induction of pseudoflashes in dead fireflies makes it unlikely that this response depends upon active participation of any part of either tracheal or nervous systems.

Even with end cell control excluded there remains the question of whether the hypoxic glow-pseudoflash response might nevertheless be controlled by oxygen limitation. We have seen that in the glow of smeared tissue and, within a narrow concentration range, in the hypoxic glow itself, oxygen does appear to be a limiting reactant. However, the hypoxic glow is actually induced not by increase in oxygen concentration but by decrease, and the pseudoflash dies away (*i.e.*, is controlled) under conditions in which ambient oxygen concentration, if changing at all, must be rising. When we add to these paradoxes the fact that glowing can be induced by pure oxygen (Alexander) and by a wide variety of physical and chemical agencies, and that even the hypoxic glow can change spontaneously in intensity without any change in ambient gas concentration, it becomes very difficult to visualize oxygen as playing any consistent role in either initiating or stopping these induced types of luminescence.

For reasons discussed by Buck (1955) the striking kinetic similarity between pseudoflash and oxygen flash (Figs. 2, 3) does not necessarily indicate the same causation. However, the detailed parallels between the two responses leave little doubt that the pseudoflash of the intact organ involves the photochemical system identified in the cell-free extract. Thus active intermediate can be presumed to accumulate in the photogenic tissue during hypoxia, and, upon readmission of air, to be concurrently oxidized and inhibited with production of a pseudoflash (see Introduction). Similarly, assuming that liberation of active intermediate would continue in the organ of a dead firefly until autolysis supervened, the ability of some dead individuals to glow and to give pseudoflashes could be explained. Furthermore, the *in vitro* system is free from all the morphological objections to end cell involvement discussed above and it is consistent with the constant duration of the pseudoflash, which is particularly difficult to account for on the basis of valvular control. Such constancy, in other words, is precisely what would be expected if the luminescence decays primarily because of an enzyme-inhibiting reaction rather than because of oxygen limitation.

The conclusion that no end cell valve functions in the hypoxic glow-pseudoflash response does not, of course, exclude the possibility that normal flashing is controlled by such a mechanism. Since, however, the induction of the pseudoflash has been the principal experimental support for belief in end cell control, the existence of a more reasonable alternate explanation of the pseudoflash leaves little ground for favoring end cell involvement in the normal flash. Furthermore, oxygen control by whatever method appears intrinsically less tenable than enzymatic control on at least two counts: First, if the flash were oxygen-limited the photogenic tissue would have to be hypoxic throughout the long interflash periods. This would be unlikely on physiological grounds, even if respiration had a lower oxygen requirement than luminescence (actually, in all forms thus far studied luminescence persists at oxygen concentrations far lower than will support any significant respiration). If, however, the normal flash were controlled by temporary reversal of enzyme inhibition, rather than by oxygen limitation, the tissues could remain fully aerated at all times. Second, the normal flash of many lampyrid fireflies is so short as to cast doubt on the possibility of control by diffusive gas transfer. Even in the relatively slow flash of *Photinus pyralis* (Fig. 11) the average rise time is 0.075 second, and the response of individual photogenic units is almost certainly much faster (Buck, 1948, p. 446). In the female of *Photuris* (Fig. 8) the rise time is not more than 0.03 second and the decay of luminescence is almost equally rapid. This shows that an efficient mechanism for oxygen removal would need to be present as well as one for suddenly supplying oxygen. *A priori*, therefore, it would be expected that an intracellular mechanism involving enzyme inhibition and activation would be better suited to the required response velocities than one involving passage of oxygen between tracheae and cell.

The fact that intact fireflies are able to extinguish their light completely between flashes, whereas low-level luminescence continues in the *in vitro* system, need not be unduly disturbing since the intact cell presumably has more efficient methods of shifting the chemical equilibria concerned and of sequestering reactants. It has been suggested (Buck, 1948, 1955) that the photogenic tissue is ordinarily kept dark by some sort of aerobic metabolic process and that light is produced only when this process is interfered with. Such a mechanism would account for both "abnormal" and normal luminescence, since it should be inhibited by very diverse agencies such as hypoxia, various poisons (*e.g.*, pure oxygen, cyanide) and anesthetics (ether)—allowing light to be produced—while at the same time forming a likely type of system to be integrated with the normal biological trigger, the nerve impulse. A tentative biochemical pathway has recently been suggested (McElroy and Hastings, 1955) by which a nerve impulse might lead to a rapid temporary increase in active intermediate concentration—*i.e.*, to a flash. Whether or not the precise mechanism suggested proves to be correct, this general type of linkage of stimulatory and response systems deserves special attention because it provides an endogenous mechanism capable of the observed rapidity and precision of photogenic control.

SUMMARY

As reported by Snell and Alexander, lampyrid fireflies exposed to oxygen concentrations of the order of 2% or lower develop a sustained "hypoxic glow," and when subsequently re-exposed to air emit a much brighter and shorter "pseudoflash." We find that these responses can be independent of the spiracles, and are given by decapitated fireflies, isolated abdomens and excised photogenic organs, showing their independence of central nervous system and tracheae. The hypoxic glow-pseudoflash response is also given by the elaterid firefly *Pyrophorus* and by the larval and pupal photogenic organs of lampyrid fireflies. Since all these organs lack tracheal end cells, these cells cannot, as Snell and Alexander believed, control this type of light production. This, together with other evidence, makes it clear that luminescence is rarely oxygen-limited. Rather, all our observations are consistent with enzyme activation and inhibition in a system of photochemical reactions of the sort proposed by McElroy and Hastings (1955).

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HOW SEA STARS OPEN BIVALVES[1]

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The damage inflicted upon the oyster and clam industries by sea star predation (Galtsoff and Loosanoff, 1939; Barnes, 1946) has stimulated much interest in the method employed by asteroids to open the shells of bivalve molluscs. The many solutions proffered in the past were reduced to two probable alternatives within the last sixty years: (1) the "toxin" theory which proposes that sea stars secrete a substance which produces relaxation of the adductor muscles of their victims; and (2) the "mechanical" theory which credits the sea stars with the ability to pull the valves of the molluscan shell apart by means of their tube feet.

The first hypothesis was proposed originally by Eudes-Deslongchamps (1826). Most of its advocates (including Hesz, 1878; Figuier, 1891; Pieron, 1913; Cahn, 1950; Korringa, 1953 and Aldrich, 1954) postulated that the chemical agent was secreted by the digestive organs of the sea stars. Van der Heyde (1922) and Sawano and Mitsugi (1932) supported this view with experiments which demonstrated that extracts of asteroid stomach and/or pyloric caeca produce tetanus and, often, permanent cessation of cardiac beat when poured over the hearts of living molluscs.

The mechanical theory, advanced originally by Fischer (1864) and Bell (1892), was established firmly by Schiemenz (1895) who demonstrated experimentally that the valves of the clam *Venus verrucosa* could be separated by a pull of 900 grams, while a clam held by the tube feet of an *Asterias* could be released only if a pull of more than 1000 grams was applied to it. He concluded that the sea star could exert a pull greater than that which could be sustained by *Venus*, but he failed to note that he had measured only the adhesive capacity of the echinoderm's tube feet. He did not show that the sea star possessed the ability to produce sufficient muscular force to open bivalves. However, it is believed that the data presented below demonstrate the existence of such forces and render the toxin theory less tenable.

MATERIALS AND METHODS

The two groups of experimental procedures employed were designed to determine (1) the effects of sea star extracts upon a representative bivalve, and (2) whether sea stars actually pull upon the valves of their prey.

1. *Procedures for determining effects of extracts*

The stomach and/or pyloric caeca of *Asterias forbesi* (obtained from the Marine Biological Laboratory at Woods Hole) were excised and ground with a Pyrex glass homogenizer in the cold. Enough sea water or distilled water was added to make up 10% solutions relative to the wet weight of the organs used. (Other concentrations were tested and, generally, yielded similar results.) Extraction was allowed to proceed for varying times (5 minutes to 48 hours) and the tissue debris was removed by filtration or centrifugation. Other extraction methods were employed to test the possibilities that the alleged toxin might be only poorly soluble in water, that it might occur in bound form, or that it might require activation. Thus, some extractions were made with fat solvents, some extracts were dialyzed, others were frozen and thawed before use, and some were mixtures of homogenates from different organs.

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[1] This investigation is a portion of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Zoology at Syracuse University in September, 1955.

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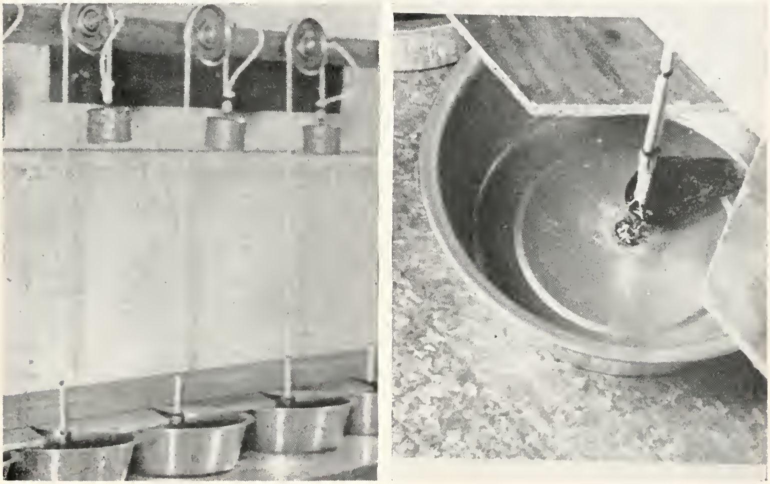
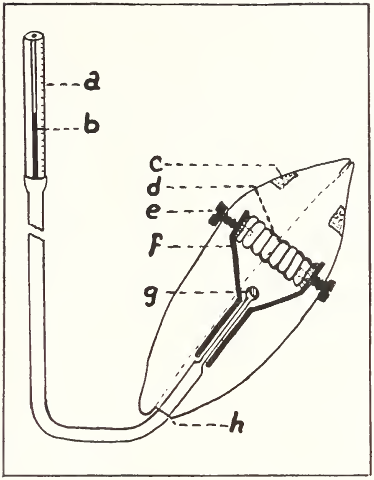


FIGURE 1. Constant stress apparatus. Each 800-gram weight was suspended by a cord passing over a ball-bearinged pulley to a double hook inserted into notches filed in the beak of the mussel shell. Another hook, also made from two bent pins, was soldered to the bottom of the pan and passed through the same notches. Gapes were measured by means of a calibrated metal triangle which could be slipped in between the valves near the hooks.

All extracts were tested on the common sea mussel, *Mytilus edulis*. In most cases 0.5 ml. of the clear extract was injected by means of hypodermic syringe into the mantle cavity or 0.15 ml. was injected directly into the posterior adductor muscle by way of a notch filed in the shell's dorsal edge. Each mussel had been pre-tested to insure that its physiological condition was approximately comparable to that of the other experimental animals. The pre-test was accomplished by exerting a pull of 800 grams on the valves for five minutes; only mussels which gaped less than one mm. were used for injection tests. After being injected, each mussel was subjected to a steady pull of 800 grams on its valves (Fig. 1) for 45 minutes during which measurements of the gape were made at regular intervals.

In some cases, the extract was merely added to the sea water into which the mussel was placed after having been kept out of water for 12 hours, and the gape was determined after 5 and 10 minutes. In other experiments, the mussel heart was exposed and perfused with the extracts while kymograph records were made of the effects on the beat. Controls for all types of tests were treated with solvent only (sea water or distilled water) or with extracts of other sea star organs or extracts of the digestive organs of other invertebrates.



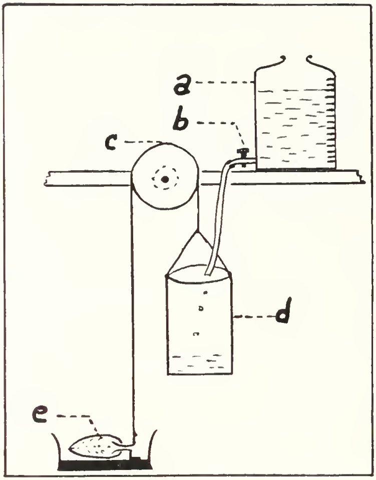


FIGURE 2. Apparatus for measuring sea star pulling force. The device and the mussel are represented at approximately actual size. *a.* calibrated capillary tube; *b.* water column; *c.* cut posterior adductor muscle; *d.* steel coil spring; *e.* bolt; *f.* metal plate soldered to the spring; *g.* plugged end of water-filled rubber tube; *h.* cut umbo. In some experiments this manometric unit was replaced by a plastic cylinder which fitted between the two bolts.

FIGURE 3. Increasing load stress apparatus. *a.* calibrated water jar; *b.* control valve; *c.* pulley; *d.* waxed cardboard container; *e.* mussel. The approximate total load applied to the shell was computed by adding the container weight to the weight of the water poured into it from the calibrated jar.

2. *Procedures for determining sea star pulling ability*

The adductor muscle of medium-sized *Mytilus* was severed with a thin razor blade and the valves were then made to shut firmly by means of an "artificial muscle." This consisted of a tightly coiled steel spring about ½ inch long with a metal plate soldered at each end. The spring was held in place (Fig. 2) by short bolts inserted through holes bored in the valves. The metal plates were bent so as to compress the sealed end of a water-filled rubber tube which passed out of the shell through a hole effected by breaking off one tip of the umbo. The distal end of the rubber tube was slipped over the end of a graduated capillary tube. Any outward pull on the valves was reflected in the stretching of the spring and, consequently, in an increased volume of the rubber tubing and a lowering of the water level in the manometer tube. The variations in water level, produced by a sea star humped over a mussel containing this apparatus, could be duplicated by inserting the mussel, afterward, in the stress apparatus illustrated in Figure 3. In some instances, the severed adductor muscle was replaced by a threaded plastic cylinder so that the valves could be bolted together firmly or allowed to separate only slightly.

Table I

Gaping of Mytilus under stress. These raw data are from two representative groups of experiments involving the application of stress to the shells after injections into the adductor muscles. The apparatus permitted the testing of 10 mussels simultaneously; generally, five were treated with extract and five with control solutions. Shells ranged in size from 43 × 22 mm. to 55 × 30 mm.

Injected with	Gape in millimeters after					
	5 min.	10 min.	15 min.	20 min.	25 min.	45 min.
Distilled water	0	0	0	0	0	0
	0	0	0.7	2.0	0.2	2.0
	0	0	4.8	4.0	5.8	7.8
	0.5	0	9.6	0.2	2.8	2.2
	0.9	2.8	1.7	1.8	2.8	1.8
	2.2	2.5	2.7	2.7	2.5	2.5
	2.8	2.9	3.8	3.0	3.8	3.2
	3.5	3.2	3.2	3.8	3.5	4.0
	3.8	4.8	4.9	5.0	5.0	5.5
	3.8	4.0	3.8	4.8	5.7	6.2
Pyloric caeca in distilled water	0	0	0	0	0	2.2
	0	0	0	0	0	4.8
	0	0	0	0.8	3.7	4.8
	1.8	1.8	1.9	3.0	3.0	2.8
	1.7	3.0	3.2	3.0	3.0	2.0
	1.8	1.8	1.8	1.8	1.9	5.8
	2.1	2.1	2.1	2.2	2.5	3.0
	3.8	3.8	3.8	5.0	5.3	5.4
	4.0	4.0	3.8	4.0	4.8	4.8
	4.8	5.7	5.8	5.8	5.8	4.8

Results

1. *Effects of sea star extracts*

As reported by previous investigators, extracts of the digestive organs of sea stars generally produce tetanus in molluscan hearts. But so do other substances including sea water. Furthermore, any suggestion that an asteroid secretion may affect the adductor muscle indirectly by stopping the heart seems untenable in view of the observation, made in some of these experiments, that *Mytilus* whose hearts are excised may continue to maintain their valves tightly shut for two or three days.

Mytilus placed in sea water contaminated with sea star extracts usually "taste" the medium and then close their valves firmly. The degree of gaping, among the sixty mussels tested in this manner, was less for specimens exposed to diluted extracts than for those placed in sea water alone. This seems to indicate that no muscle-relaxing toxin was present in the extracts.

Gape measurements made on mussels injected with extracts or control solutions revealed that the rate of shell opening varied through a very narrow range for all tests. The average value of the rate of gaping for mussels which were not injected was almost identical to that of mussels whose mantle cavity or adductor had been injected with sea water or distilled water or with one of the various types of extracts (see representative data in Table I). Over 1000 mussels were tested in this manner and the data can only lead to the conclusion that the extracts did not contain any substance which could be considered effective in inducing relaxation of the bivalve adductor muscles.

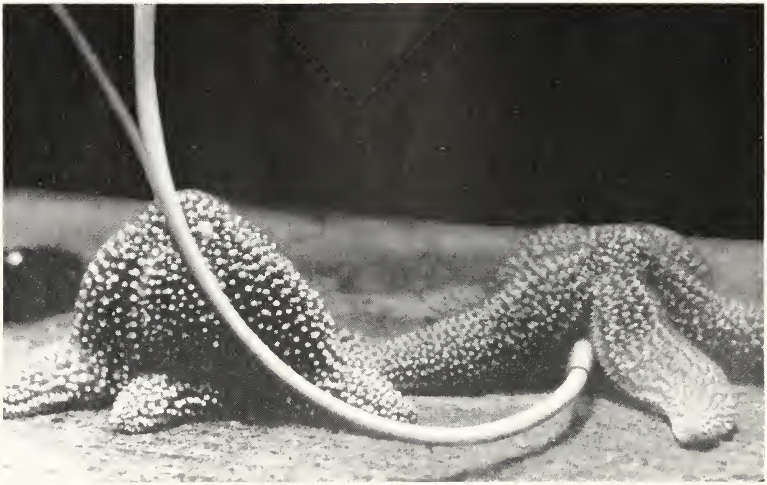


FIGURE 4. *Asterias* feeding upon decoy mussels. The rubber tube leading to the manometric recorder is covered with a glass sleeve near the mussel in order to prevent compression of the tube by the sea star's antimeres. The asteroid on the left is in the process of inserting its stomach into a shell whose valves are tightly bolted together by means of plastic cylinder.

2. *Observation of sea star pulling ability*

Sea stars, kept in 20-gallon tanks of circulating sea water, were presented live mussels whose adductors had been replaced by springs or cylinders as described previously. The soft parts of most of these mussels were reached by the asteroid stomachs and were partly or wholly digested. Unquestionably, no secretion of the sea stars could have had any weakening effect upon the "artificial muscles" holding the pelecypod valves closed. The following cases, selected from several dozen observations, illustrate the significance of the results obtained:

1) A sea star was observed while it approached a mussel containing the spring device and while it humped over its victim in the typical predatory position (Fig. 4). During the five minutes it required to settle in an advantageous position (and, probably, to extrude its stomach) there was no change in the water level of the recording apparatus. During the next three minutes, however, the level dropped rapidly; at the end of this time the sea star was removed from the aquarium and its arms were peeled back forcibly in order to expose the mussel. The valves were found closed tightly upon the sea star's stomach, most of which was inserted into the shell. In this case, the drop in the recording tube was duplicated later with a load of 1200 grams on the shell's valves; but spring-containing mussels requiring 2600 to 3000 grams pull to open 0.1 mm. were also successfully preyed upon by the sea stars.

TABLE II

Summary of pulling forces exerted by a sea star upon a spring-containing mussel

Time (in minutes) from beginning of observation	Pulling force (in grams) applied by the sea star
0	440
5	740
10	620
14	710
15	560
20	620
60	470
90	0
115	650
135	0
150	680
155	800
158	0
159–165	Sea star moved off mussel

2) Another *Asterias* was observed for almost three hours after it was found humped over a prepared mussel. During that time, the water level of the recording tube varied through three irregular cycles of rises and falls. When these variations were duplicated later by placing the mussel in the stress apparatus, it was seen that they represented the pulling forces shown in Table II. When the mussel was opened it was found to be partly digested. This, and many similar observations, seems to indicate that the sea star's pull is not applied steadily.

3) A mussel whose valves were bolted together very firmly so that no space could be discerned between them under 9 × magnification, was loosened forcibly from the grasp of a sea star that had humped over it for several hours. The asteroid's stomach was mostly inside the shell and it did not slip out again during the next hour while the sea star dragged the shell along the bottom of the aquarium. Later, when the shell was exposed to increasing loads in the stress apparatus, the valves were bent enough by a load of 3100 grams to produce an opening between them of 0.1 mm.

4) Several mussels whose valves were tied together so as to open only 0.1 mm. were invaded by sea stars whose stomachs were seen to slip out of the shells when the echinoderms' arms were pulled away from the shells.

Discussion

The negative results of the experiments involving sea star extracts are not proof that asteroids do not secrete a toxin during predation, but they do indicate that no such substance can be separated from the sea star organs by the extraction methods used. Furthermore, a muscle-relaxing secretion would seem superfluous, at least in the predation of *Asterias forbesi* upon *Mytilus edulis,* since it was shown above that this asteroid is capable of producing a pulling force which is transmitted to the valves of mussels by the anchoring action of the tube feet.

It may be questioned whether some species of pelecypods which are attacked by sea stars might not require stronger pulls to open than those that can be mustered by *Asterias.* Reese (1942) showed that 3750 grams could be withstood for several days by some *Venus* and *Ostrea;* Tamura (1929) reported that the Japanese oyster may sustain 15,000 grams pull for as long as five minutes; Galtsoff (1952) referred to the ability of oysters to withstand 6000 grams for several hours; Plateau (1884) computed *Ostrea's* "absolute resistance" (equal to the force required to open its shell one mm.) at 5026 grams, while Marceau (1909) reported that *Mytilus* could withstand a pull of 11.3 kg./sq. cm. of its adductor muscle tissue.

These impressive figures seem to preclude any possibility that sea stars pull open the shells of *Ostrea* and *Venus.* But, on closer examination, Plateau's "absolute resistance" appears outstandingly significant—if a force of 5026 grams can produce an opening of one mm. in *Ostrea,* might not a lesser pull be sufficient to open the shell 0.1 mm., the smallest measured gape through which sea stars' stomachs have been observed to penetrate? Many of the objections to the mechanical theory in the past have been based on the supposition that much larger gapes would be required (Reese proposed 7 mm. as a minimum), and the fact that such wide openings could be effected only by tremendously strong forces which a sea star could not be expected to exert. The experimental results described above have shown that *Asterias* is capable of producing pulling forces equivalent to 3100 grams. It seems likely that even greater forces could be demonstrated with adequate apparatus. Therefore, there is little reason to suppose that the usual bivalve prey of sea stars cannot be opened by the attached tube feet, at least enough for the insinuation of the stomach. According to this view, only very large and highly resistant molluscs would be immune to sea star predation. In fact, the larger, more resistant *Mytilus edulis* are seldom attacked successfully by sea stars. However, Feder (1955) reports that the larger *Mytilus californianus* are eaten by asteroids, but that entry into the shell is gained by way of the mussel's byssus "door" which is relatively wide in that species. By contrast, only one among the hundreds of east coast *Asterias* observed during this research was seen to have employed this approach. Feder also measured forces and shell openings which closely approximate the figures reported herein.

It must be emphasized that the observations made during this investigation do not support the popularly accepted notion that the process of predation is a "tug-of-war" in which the sea star becomes the victor by virtue of its persistence and greater endurance. The penetration is effected, as shown above, quite rapidly and as the result of a sudden overwhelming force, which is relaxed and re-applied at intervals until digestion of the soft parts of the bivalve has proceeded to the point where the adductor muscle is rendered ineffective.

The exact mechanism responsible for the pulling force has not been established. However, it is thought to reside in the musculature of the tube feet described in detail by Smith (1937, 1947). Once humped over the bivalve, the asteroid's body moves very little or not at all, but the tube feet are very active, protracting and retracting in such a way that they give the impression of operating in relays. Each tube foot's muscular tissue is ample to overcome the 29 grams of adhesiveness of the base (Paine, 1926). If this value is used as a criterion, then, it would appear that a sea star would need to employ less than one-fourth of all its tube feet simultaneously to produce pulls of over 5000 grams.

SUMMARY

1. An investigation was made into the possibility that sea stars secrete a substance which is toxic or anesthetic for bivalves. Extracts prepared from the organs of feeding and non-feeding *Asterias forbesi* were introduced into the adductor muscle and the mantle cavity, or perfused over the beating heart, of *Mytilus edulis*. The effects of such solutions were, generally, identical to those produced by sea water or distilled water.

2. Sea stars were induced to feed upon specially prepared mussels, so that the forces which their tube feet exerted on the shells could be measured manometrically. The adductors of the mussels used in such experiments had been severed and replaced by steel springs or plastic cylinders which could not be affected by any alleged toxin. It was found that the tube feet did pull the valves apart and forces of over 3000 grams were recorded. It was observed also that a very minute opening between the valves (0.1 mm.) was sufficient to permit the insinuation of the asteroid stomach.

3. The common interpretation of the mechanical theory, which asserts that the sea star "fatigues" the mollusc, appears inaccurate in view of the findings of this research. There is evidence that the opening of the valves is a rapid process involving overwhelming, discontinuous forces, so that the predator may be considered to relax its pull upon the valves at intervals and to allow its stomach to be compressed between the valves until it pulls them apart again.

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STUDIES ON MARINE BRYOZOA. VIII. EXOCHELLA LONGIROSTRIS JULLIEN 1888

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The writer wishes to express deep gratitude to the National Science Foundation for research grants aiding this and other studies and to the Smithsonian Institution, U. S. N. M., for the loan of bryozoan specimens collected during the U. S. Navy's 1947–48 Antarctic Expedition by Comdr. David C. Nutt.

The purpose of this study is to report *Exochella longirostris* Jullien 1888 (order Cheilostomata, Family Exochellidae) from the Antarctic, to raise some questions about its synonymy and to add further morphological and ecological data to the limited information existing on this species.

Exochella longirostris Jullien 1888

(Figures 1 A–J)

Synonymy and distribution data:

1888. *Exochella longirostris.* Jullien pp. 55–56, Pl. 3, Figs. 1–4; Pl. 9, Fig. 2. From Ile Hoste, baie Orange, Canal du Beagle, Ile Gable, Tierra del Fuego. 19 meters.
1904. *E. longirostris.* Calvet p. 29. Magellan Straits, Punta Arenas.
1908. *E. longirostris.* Canu p. 300, Pl. VI, Fig. 13. From Post-Pampeen de Punta Borja, Puerto Militar, Bahia Blanca (Argentina).
1937. *E. longirostris.* Marcus pp. 82–83; Pl. 17, Fig. 43. Bay of Santos, Brazil; 20 meters.
1941. *E. longirostris.* Marcus p. 22; Fig. 16. Sta. Catharina, Parana; Guaratuba.
1949. *E. longirostris.* Marcus p. 1. South of Victoria, Espirito Santo, Lat. 20°33'S., Long. 40°14'W.; 35 meters.
1952. *E. longirostris.* Mawatari p. 265. Wakayama Prefecture, Shirahama and Tonda, Japan.

Some difficulty was encountered in the identification of this species because Jullien's original description was inadequate. Externally, the USNM specimens resemble those pictured by Jullien (1888) and Canu (1908) but these authors did not figure the internal aspect of the primary orifice, a very important diagnostic character. Levinsen (1909, p. 321; Pl. 17, Figs. 6a–c) beautifully and completely described an *Exochella longirostris* from Challenger Sta. 315, Falkland Islands. However, it is not at all certain that Levinsen and Jullien were describing the same species. Levinsen pictured a distinct lyrula on the proximal border of the primary

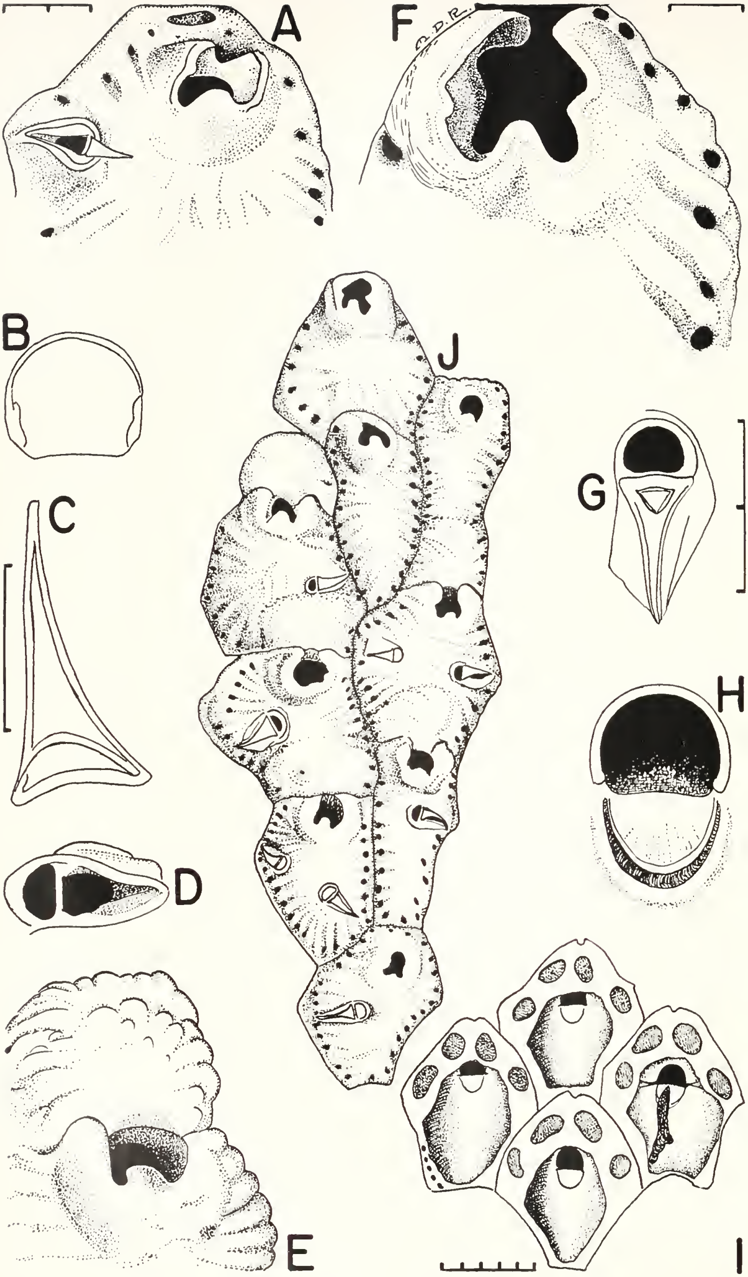
A
F
B
J
G
C
H
D
E
I

FIGURE 1, *A–J.*

orifice, while Jullien stated that the orifice is rounded and that its peristome is prolonged forward and backward. The USNM specimens do not show such a prominent structure so immediately within the primary orifice but do show the peristome thickened medially to simulate a lyrula a little in front of the primary orifice border. Waters's notations on this species (1889 *E. longirostris* and 1906 *Smittia longirostris*) are not precise enough for one to be able to determine if he actually had Jullien's species, so are not included in the present synonymy. Marcus' Bay of Santos specimens are considerably smaller in all parts (zooecia, apertures, ovicells, avicularia) than the USNM specimens, judging from the scale accompanying Marcus' Pl. 17, Fig. 43. His avicularia seem much thinner than those of the present specimens. Finally, the peristomial processes appear to be thinner and sharper than those of the USNM material. In spite of these differences, it is believed that these belong to the same species and that the differences are due to ecological and geographical factors, the USNM Antarctic specimens showing the sturdiest and largest specimens of this variable form.

Diagnosis: Zoarium encrusting, heavily calcified. Zooecial boundaries distinct. Convex frontal an areolate pleurocyst, somewhat ribbed in old zoids. Ovicells nonporous, covered over by the frontal of the next distal zoid. Avicularia adventitious, pointed, medium-sized, frontal, not peripheral nor over an areolar pore; one, two or none may occur on a zoid. Mandible long, triangular, sharply pointed. Peristome incomplete distally in ovicelled zoids. Raised peristome develops a mucro, sometimes medially thickened to simulate a lyrula. Peristomial sinus on each side of the mucro. Peristomial side walls raised, sometimes pressing inward. Primary orifice has a hemispherical vestibular arch. Immediate lyrula and cardelles absent.

All figures are drawn with the aid of a camera lucida.

FIGURE 1. *Exochella longirostris* Jullien 1888, from the Antarctic.

A. A zooecium at growing edge of colony. Thin young peristome still incomplete in back, with two sinuses and a lip-like mucro proximally. Mandible opened. One dietella (broad distal pore) shown at top. Drawn to the 0.2 mm. scale above.

B. Operculum, drawn to the Figure G scale.

C. Mandible, drawn to the 0.1 mm. scale at left.

D. Avicularium with membranous part burned off. Drawn to the Figure G scale.

E. An old ovicelled zooecium. The very thick peristome is worn away in front and at right. The projection simulating a lyrula is not a true lyrula but a thickened "core" of the mucro, a thickening of the proximal peristomial wall and characteristic of the most heavily calcified zooecia. Drawn to the Figure A scale.

F. Another young incomplete peristome. The mucro is more pointed and the side walls press in more acutely than in Figure A. Drawn to the 0.1 mm. scale above.

G. An avicularium with mandible in place. Membranous "back" area in black. Drawn to the 0.2 mm. scale at right.

H. The primary orifice in black, vestibular arch above, and compensation sac area below, as seen from inside the zooecium. Drawn to the Figure F scale.

I. Four zooecia seen from the attached basal side. Each has four dietellae (large heavily stippled distal pores). Five small areolar pores are on the lower corner of the left zoid whose basal wall has broken away there. The zooecium at right has the remains of the operculum and tentacular sheath suspended from the orifice. Compensation sac area also plainly visible in all. Drawn to the 0.5 mm. scale below.

J. Nine old zooecia. The upper three and the lowest one are non-ovicelled. The remaining five have non-porous ovicells more or less undistinguishable from the frontal of their distal zooecia. They can be recognized by the incomplete distal wall of the peristome. One, two or no avicularia may be present per zooecium. They are not areolar but are more central in location. Drawn to the Figure I scale.

Operculum forms three fourths of a circle, with proximal edge bevelled. Compensation sac about the size of the primary orifice. Three to five dietellae.

Measurements. The first figures are the minimum, the next the maximum and the last, in parentheses, the average of 10 readings for each structure (except for the avicularia whose averages are based on 30 readings). Length and width are abbreviated to L and W. Readings are in millimeters.

0.734–0.979	(0.888)	Zooecia L
0.605–0.922	(0.736)	Zooecia W
0.158–0.259	(0.204)	Avicularia L
0.072–0.130	(0.102)	Avicularia W
0.115–0.147	(0.131)	Primary orifice L
0.144–0.166	(0.155)	Primary orifice W
0.144–0.173	(0.153)	Secondary orifice L, including sinus
0.101–0.144	(0.124)	Secondary orifice L, exclusive of sinus
0.144–0.173	(0.153)	Secondary orifice W
0.302–0.360	(0.334)	Ovicell L
0.360–0.418	(0.382)	Ovicell W
0.137–0.158	(0.147)	Operculum L
0.130–0.173	(0.154)	Operculum W
0.128–0.151	(0.137)	Mandible L
0.058–0.073	(0.068)	Mandible W
0.115–0.158	(0.130)	Compensation sac area L
0.122–0.158	(0.143)	Compensation sac area W

Zoarium. The ivory-colored, heavily calcified zoarium is sturdy and sometimes extensive. A 25 × 36-mm. pebble had one surface completely encrusted by one colony. Colonies are unilaminate, forming a thick crust, usually numbering many zoids. Polypide remains present in some.

Zooecia. The hexagonal zooecia are distinct and sizable. Some are ovicelled, some not; some have avicularia, others do not. From the basal aspect (Fig. 1, I), the three distal walls are convex, the three proximal walls concave. The thick frontal is a granular to beaded pleurocyst. Ridges arise between its closely spaced elliptical areolar pores and continue part way up the frontal (Fig. 1, F, J). The compensation sac area is small and immediately below the orifice (Fig. 1, H, I). The basal, attached zooecial surface has 3 to 5, usually 4, large oval dietellae (Fig. 1, I).

Avicularia. One or two frontal avicularia occur on many of the zoids. Their orientation is variable on the solid part of the frontal. They are not oral nor areolar though some occur fairly close to the zooecial edge. Others are more central (Fig. 1, J). The small avicularial chamber tips the beak upward along a modest slope. The avicularia are always of the same type and of fairly uniform size. Their back area is hemispherical, the beak triangular and longer. The mandible is a narrow triangle, with the two long sides concave (Fig. 1, C, G), and edges reinforced. The USNM avicularia, though larger in actual measurements than those of Marcus' species, are smaller in proportion to the rest of the zooecial front than are Marcus' specimens.

Orifice. The orifice is not terminal but a slight distance short of that. Its distal wall is not formed by the next distal zoid. The deeply set primary orifice is slightly more than hemispherical, with a handsome vestibular arch and a nearly straight proximal border (Fig. 1, H). The chitin-rimmed operculum has the same shape (Fig. 1, B). Lyrula and cardelles are absent in the primary orifice but the peristome immediately in front of the operculum simulates a lyrula. This appears to be at variance with Levinsen's figures which show a lyrula apparently right on the border of the primary aperture. Whether or not this is the condition of Jullien's original material is unknown. The secondary orifice shape is variable, depending on the degree of calcification, being sometimes trifoliate, sometimes horseshoe-shaped (Fig. 1, E, F, J). The distal peristome wall is entire in mature non-ovicelled zoids but interrupted by the ovicell in fertile ones. The peristome thickens considerably with age. Proximally the peristome develops a tab-like mucro (Figs. A, F) bordered on each side by a sinus. The mucro may thicken medially to such an extent inward that it could be easily mistaken for a lyrula (Fig. 1, E). Laterally, the peristomial wall may or may not pinch in (Fig. 1, F).

Ovicells. Young ovicells are salient, old ones heavily calcified and immersed. They are not porous but some are bordered laterally by a few areolar pores which do not penetrate the ovicell wall proper. The ovicell surface is granular to beaded, occasionally irregularly ridged (Fig. 1, E). No avicularia occur on the ovicells nor does the peristome encroach upon them but the frontal of the next distal zoid covers the ovicell front completely.

Distribution and ecology. This species' most northerly record (and the only one for the northern hemisphere) is that of Mawatari (1952) from Japan. All other previous records are from the southern hemisphere, ranging from 20°33'S. Lat. (Marcus, 1949, south of Victoria, Brazil) to about 55°40'S. Lat. (Jullien, 1888, Tierra del Fuego, Ile Hoste).

The USNM specimens appeared on a rock from Sta. 184 and on pebbles Nos. 2, 3, 4, 12, 13 and 16 from an unidentified Antarctic locality (Comdr. D. C. Nutt, U. S. Navy's 1947–48 Antarctic Expedition). Station 184 was at Marguerite Bay, Antarctica, location approximately 68°30'W. Long. and 68°30'S. Lat., bottom dredge haul, depth 85–100 fathoms, water temperature 30.2° F., Feb. 19, 1948. This represents the most southerly and deepest record for the species and the first time it was collected well within the Antarctic Circle. Some of the USNM colonies have grown over Foraminifera, incorporating their shells within the zooecial base. Sponge spicules are matted over one colony, calcareous worm tubes and occasional bryozoan zoids (of other species) are present on other colonies. However, most of the colony surface is free of extraneous growths. The Antarctic specimens appear to be much larger, thicker-walled and more sturdy than those from warmer localities. The present study specimens are on deposit at the U. S. Nat. Museum, Smithsonian Institution, Cat. Nos. 11325, 11326, 11327 and 11328.

Affinities. Exochella longirostris Jullien 1888 and a fossil species *E. grandis* Canu and Bassler (1935, p. 32, Pl. 9, Fig. 3) from the Tertiary Balcombian Beds of Muddy Creek, Victoria, Australia, appear to be closely related. The USNM specimens are similar in size and measurements to *E. grandis* but lack the prominent mural thread and the very conspicuous beading of the pleurocyst. In *E. grandis* the avicularia replace the areolar pores but in the USNM *E. longirostris* they generally do not and are less peripheral.

SUMMARY

1. The geographic range of *Exochella longirostris* is extended to the Antarctic.

2. The Antarctic specimens are sturdier, larger and thicker-walled than those of the same species from warmer waters and have avicularia which are a bit smaller proportionately, although larger in actual measurements.

3. Numerous measurements of various structures and zooecia are included, to show the range of variation for this species.

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MICROGEOGRAPHIC VARIATION AS THERMAL ACCLIMATION IN AN INTERTIDAL MOLLUSC

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A growing body of literature suggests that many poikilotherms are able to regulate, to a remarkable degree, their physiological activity rates. Regardless of the latitude over which certain species are distributed or the seasonal temperature change to which they are subjected, their physiological rates converge towards a mean value. To accomplish this relative constancy, northern populations and winter forms often have higher rates of activity, metabolism and development than southern populations and summer forms when measured at the same temperature. Animals thermally conditioned in the laboratory have also shown this compensatory phenomenon.

Ample documentation is provided in the comprehensive review of Bullock (1955), who also presents data showing temperature adaptation at the tissue, cellular and enzyme level, and in that of Prosser (1955), who summarizes evidence of compensatory adjustment to oxygen tension, osmotic pressure and drugs, as well as to temperature. Roberts (1952) and Dehnel (1955), who themselves have contributed studies of this problem, give additional references.

In 1953, Segal, Rao and James extended the known cases of intraspecific physiological differentiation with respect to temperature to include microgeographically separated individuals of the species. The heart rate in the limpet *Acmaea limatula* and water propulsion in the mussel *Mytilus californianus* were faster in samples from low intertidal levels than from high levels at any given temperature.

In the present study, an attempt has been made to corroborate and extend the initial findings on *A. limatula* and to ascertain whether the significant parameter of the difference in microhabitats is temperature. Besides heart rate, differences in gonad size and spawning readiness have been investigated. A major portion of this study constitutes an attempt to test the hypothesis that we are dealing with individual adaptations to habitat temperature.

MATERIALS AND METHODS

Habitat

The aspidobranch gastropod *Acmaea limatula* Carpenter is a eurytopic intertidal species which at Palos Verdes, California (Lat. 33° 43′ N., Long. 118° 16′ W.) has a vertical distribution of approximately 1½ meters from a mid-tidal to a low-tidal level. Highest and lowest individuals may be separated by as much as 20 meters

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[1] The work was performed in the Department of Zoology, University of California, Los Angeles, Calif.

[2] I wish to thank Dr. T. H. Bullock for his encouragement and guidance throughout the course of this investigation.

of sloping, rocky beach; the nature of the beach prohibits interchange between higher and lower levels.

At each of four collecting sites (referred to in the text as sites 1, 2, 3, and 4) the low-level specimens were taken from below zero datum (mean lower low water: U. S. Coast and Geodetic Survey Tide Tables, Pacific Coast) where they are at the temperature of the surf but for a few hours each month. The higher level specimens were taken 1 to 1½ meters above zero datum where they are subject to exposure about 50% of the time.

Collection and care of animals

Animals were removed from the substrate with a thin spatula and transported wet to the laboratory in enamel or plastic trays. In the laboratory the animals were covered with fresh sea water, aerated, and refrigerated at temperatures approximating the average ocean temperature for that season (see Fig. 7). Each day the water was replaced with fresh sea water at the same temperature.

During the winter and spring months an attempt was made to approximate the natural exposure time for high-level individuals. The water was poured off and the animals allowed to warm to room temperature and stand for 5–6 daylight hours.

Heart exposure

No later than 24 hours after collection, the heart was exposed by cutting a hole in the shell to the left and slightly posterior to the shell apex with a fine toothed trephine. This tool was designed to take different sized cutting heads: 3 mm. in diameter for cutting small shells, 4½ mm. in diameter for cutting larger shells. A retractable pin in the center of the trephine prevented the cutting edge from wandering. The surface of the mantle exposed by the hole was flushed clean of shell particles with a fine stream of sea water.

Each animal was numbered with colored lacquer, then placed in a 10-inch finger bowl (15 to 20 individuals per bowl) which contained 1–1½ inches of sea water. The animals were returned to the refrigerator for one–two days to allow the gut to empty and permit recovery of the animals from any possible operative shock.

Recording procedure

The day heart beats were counted, two finger bowls were placed on a wire mesh platform two inches below the surface in a 15-gallon aquarium. Water circulated through the aquarium at a constant temperature ± 0.2° C.

Initially, the water bath was at the refrigerator temperature and was then gradually lowered to 4°, 7°, or 9° C. Two to four hours were allowed for the animals to reach the lower temperatures. Temperatures were raised by increments of 5° C. to a maximum of 29° C. Animals were allowed two hours to reach each temperature.

Using a stop-watch, the number of seconds required for 10 heart beats was counted by eye. At lower temperatures a reading was taken of each animal in the group and this was repeated a second and third time. At higher temperatures, because of the greater possibility of error due to the increased heart rate, the readings were taken 5 times. Plotted points (see Figs. 1, 3, and 4), are the average of these data converted to beats per minute.

The basic measurement used in this study is stable and reliable. A few minutes after the operation, which does not break the mantle, the heart rate settles to a value which is consistent over many hours and even days. Those animals showing excessive locomotor activity or irregularity of heart activity were discarded.

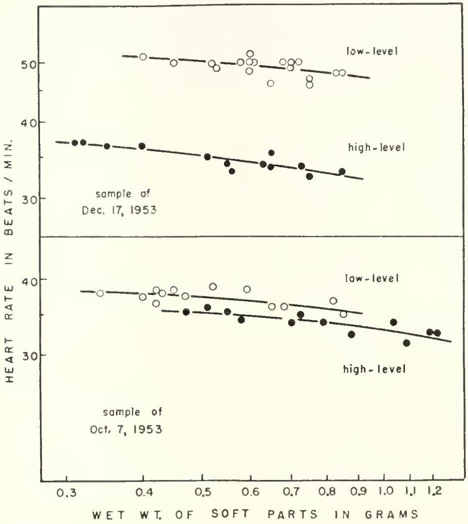

FIGURE 1. Relation between heart rate and wet weight of soft parts at 14° C. for vertically separated *A. limatula* from Palos Verdes. Points are averages of 3 to 5 readings of the number of seconds per 10 beats for one individual. The December collection shows a near maximum excursion of the difference in heart rate between samples. The October collection shows the minimum difference. Co-ordinates are logarithmic; equal percentage deviation is, therefore, shown by equal spatial spread from the hand-drawn regression lines anywhere on the graph. All curves are eye-fitted.

RESULTS

Effect of Intertidal Height on Heart Rate

When the animals' heart rates are measured at temperatures from 4°–29° C., it is found that the lower in the range of distribution an animal lives the faster is its heart rate. A comparison is made of the heart rates of "highs" and "lows" and the near maximum and the minimum difference in rate between samples are illustrated in Figure 1. At lower temperatures the absolute difference in heart frequency between "highs" and "lows" is less than it is at higher temperatures.

When heart rate is plotted against temperature, it can be seen that the curve for a low-level animal is above that for an equal weight individual from high-level

(Fig. 2). Since heart frequency will be shown to vary with habitat and season, rates at successive collections could not be combined. The pair of rate/temperature curves in Figure 2 have been selected from the many pairs calculated because they show the response both over the greatest range of temperatures and the largest number of temperature points.

When both groups are tested at any temperature, within the physiological ranges of temperatures of the species, heart rates of low-level animals are as much as 30 to 40% faster than those of equal weight animals from high-level. Heart rates of equal value for "highs" and "lows" are obtained when the high-level individuals are measured at temperatures 1°–5½° C. above that of low-level individuals. In February, for example (not graphed), a low-level individual of average weight (0.6 gm.) has a heart rate of 53 beats per minute at 14° C. An average high-level individual of equal weight shows the same rate when measured at 19.5° C.

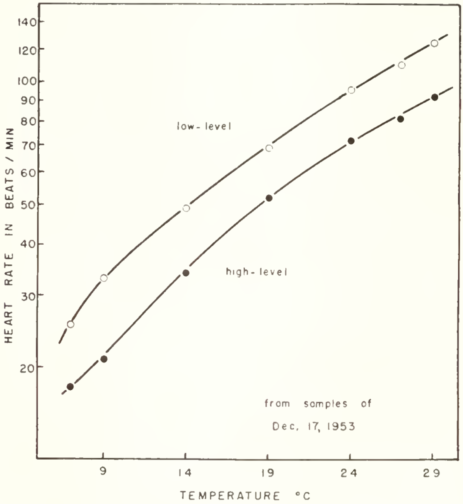

FIGURE 2. Heart rate as a function of temperature for equal weight (0.6 gm.) *A. limatula* from December collection of high- and low-level samples. Points represent intersection of perpendicular, erected at 0.6 gm., with weight regression curves at each temperature.

In addition to the difference in position of the rate-temperature curves, there appears to be a reliable difference in the slopes as measured by the Q_{10}. Between 9° and 19° C., perhaps even above 19° C., the Q_{10} of the heart rate is consistently lower for low-level animals during the winter and spring months (Table I).

It would be of value to be able to state conclusively whether high-level individuals of the species are living at a warmer temperature than low-level individuals, at least within the local coastal area. An attempt will be made to establish this point.

High-level individuals are submerged approximately 50% of the time. During the hours of exposure these individuals are subjected to air temperatures which fluctuate about the prevailing water temperatures. A series of readings (taken with a thermistor probe, one mm. in diameter, inserted under the foot of limpets in place in the field) on a sunny day in late October show that body temperatures reached a high of 30° C. in the sun and 21.5° C. in the shade. Body temperatures may have reached higher values since the animals were exposed for an additional two hours. Over a 20-year period (U. S. Weather Bureau, personal communication) 40 to 50% of the days, from October through April, have been sunny at the local beaches. From November through April the surface water temperature averages slightly over 14.5° C.

TABLE I

Q_{10} values of the heart rate for 0.6 gm. high- and low-level A. limatula over the temperature ranges indicated. Values calculated for equal weight animals on the weight regression curves at each collection period

Date	Temp. ° C.	Q_{10}	
		High-level	Low-level
7/27/53	9–14	3.40	3.14
	14–19	2.56	2.53
	19–24	1.99	1.88
10/7/53	9–14	3.22	3.10
	14–19	2.38	2.89
	19–24	1.73	1.77
12/17/53	9–14	2.62	2.30
	14–19	2.34	1.98
	19–24	2.02	1.97
1/4/54	9–14	2.70	2.49
	14–19	2.18	2.16
	19–24	1.80	1.62
2/1/54	9–14	2.57	2.34
	14–19	2.54	1.97
	19–24	1.77	1.80
2/15/54	9–14	2.67	2.54
	14–19	2.38	2.05
	19–24	1.83	1.95
4/6/54	9–14	2.74	2.37
	14–19	2.02	1.78
	19–24	1.59	1.73
5/8/54	9–14	2.51	2.37
	14–19	2.11	2.02
	19–24	1.76	1.65

Reference to Figure 8 shows that the annual range of the inshore surface water temperatures averages approximately 7° C. Air temperatures, taken a few feet above ground, show daily, and therefore monthly, fluctuations exceeding the yearly temperature range of the inshore waters. Of course, air temperatures a few feet above ground give only a rough directional estimate of microhabitat temperatures one cm. above ground. Over a period of 1½ years, dry bulb recordings have shown the microhabitat temperatures to be consistently higher than prevailing air temperatures.

Minimum air temperatures falling below ocean temperatures are encountered primarily from late spring to early autumn when low tides generally expose high-level animals during late evening and early morning hours. During the part of this period when the ocean temperature is above 17° C., the difference in heart rate between high- and low-level animals is at a minimum. The critical exposure occurs during winter and early spring in the late morning and afternoon hours. During this period the difference in heart rate is maximal.

It is of considerable interest, in this regard, that high-level animals show lower Q_{10}'s of the heart rate between 7° and 9° C. than low-level animals (low-level = 3.55, 3.66; high-level = 2.00, 2.15—two experimental recordings). It suggests that low-level animals are approaching cold depression at a higher temperature than are the high-level animals. The physiological temperature range is therefore believed to be wider for the relatively warm adapted high-level animals.

Influence of Certain Variables on Heart Rate

Body size. Among the numerous factors bearing influence upon physiological rate functions, size has been found to contribute to the variation in heart rate in the species under investigation. Size has been measured by the wet weight of soft parts. Within the weight range of 0.3 to 1.2 gm., larger animals show consistently slower rates at all temperatures from 4° to 29° C.

The regression of rate with weight is without apparent systematic variation over the year and is not significantly different between "highs" and "lows" at 14° C. ($P = .35$ for the difference between mean regression coefficients of 11 high-level and 12 low-level samples). Ten of the 23 samples show no reliable difference in regression from 9°–24° C. The remaining 13 samples have larger negative b values on either side of 14° and 19° C. Hence, no single expression is available to describe fully the effect of weight on heart rate in this species.

Within the weight range of 0.4 to 1.0 gm., the regression is usually linear when plotted on logarithmic coordinates and varies from -0.043 to -0.172. However, it is non-linear on either side of this weight range. It is as if we were plotting only a segment of a large parabola (see Figs. 1 and 3).

Both the factors of size and individual variation contribute to the scatter about each regression line. Size, however, is the major factor producing the scatter. Since weight and rate are inversely related, rate differences are meaningful only with essentially equal weight animals.

Sex. Sexes in *A. limatula,* as in all species of the genus, are separate, but three rather than two sexual states are present. To the conventional male and female is added the condition of indeterminacy. The latter is simply the post-spawning phase of the male or female in which gametes are absent (see section on gonad size).

It has not been possible to find a differential effect upon the heart rate that can be attributed to any of the sexual states excluding the pre-spawning animals heavy with gonads (see below).

Gonad size. An analysis of possible reproductive patterns will be presented in a following section. It is obvious from the data that the size and condition of the gonads vary over the year and between vertically separated individuals. Size of the gonad as such has a negligible effect on the heart rate; the condition of the gonads is, however, of importance.

On occasion when as many as 50% of a sample were possessed of insignificant gonads or were of indeterminate sex, and the remainder showed gonads weighing up to 20% of the body weight, the heart frequency of all animals fell within the scatter of either group. On the other hand, pre-spawning buildup of gonadal tissue, regardless of the size attained, rendered the heart beat erratic and not reliably measureable under our conditions. This was found in July for both high- and low-level individuals. In the latter part of the same month, when comparing heart rates of samples having approximately equal gonads by weight, high-level individuals showed slower rates consistent with the difference between groups throughout the year.

Diurnal rhythms. Two groups of 5 animals each were maintained at 14° C. and the heart beat counted at one-hour intervals over a 20-hour period. The animals were under constant illumination. Under these conditions, the presence of a day-night rhythm could not be demonstrated among either the high- or low-level individuals.

Effect of Transplantation on Heart Rate

Twenty-nine days. During March, 1953, 42 high-level and 42 low-level limpets were reciprocally transposed at site 1. Fifty control specimens from each level were handled in like manner but returned to their natural positions (see section on behavioral response). The numbers of recoveries are presented below.

Individuals transposed from low-level to high	52%
Individuals transposed from high-level to low	57%
Control individuals from high-level	84%
Control individuals from low-level	60%

Twenty-nine days after reciprocal transplantation, the heart rate, when measured from 4°–29° C., appeared to have undergone a complete reversal. Figure 3 shows the heart rate response of the transplants and controls at three selected temperatures. Heart rates of high-level individuals introduced into the low-level tide pool show a remarkable degree of overlap with those of the tide pool controls. An exception may be noted in the case of three individuals above 1.00 gm. in weight. However, since the rates for these animals fall within the variation of all transplants about the regression line, no significance has been attached to them.

Tide pool individuals transposed to high-level show heart rates close to but consistently faster than those of the high-level controls. Since animals transposed to the high-level position moved from their sites of placement to more protected positions (see below), the migrants have acclimated to a temperature somewhat lower than the "living" temperature of high-level controls.

Fourteen days. During January, 1954, a second reciprocal transplantation was performed at site 4. Recovery was exceptionally poor. Of 50 high-level individuals transposed to low-level and 50 low-level controls, all but 2 and 5, respectively, were lost. Of the same number of low-level individuals transposed to high-level, 20% were recovered as compared with 50% of the high-level controls. Heart rates of low-level controls at the end of the experimental period and high-level transplants to low-level were therefore not available for comparison in significant numbers.

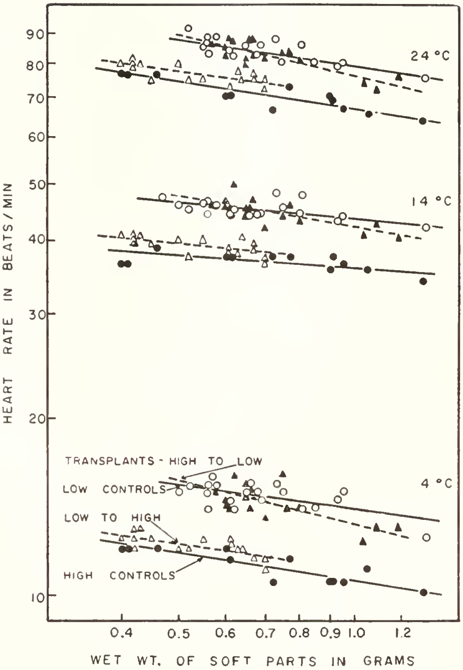

FIGURE 3. Relation between heart rate and wet weight of soft parts of reciprocal transplants and controls 29 days after transplantation. Each point represents one individual.

Comparing animals of equal weight, it is evident that the transplants to high-level have a lower heart frequency than initial low-level controls and a higher frequency than high-level controls at any temperature from 9°–24° C. (Fig. 4). The degree of acclimation of the transplants cannot be stated in equivalent °C., since the thermal history in the field cannot be given in simple terms. We only know that partial acclimation has occurred.

If we calculate the change in heart frequency of the transplants as a percentage of the difference in the frequencies of the low- and high-level controls, at the begin-

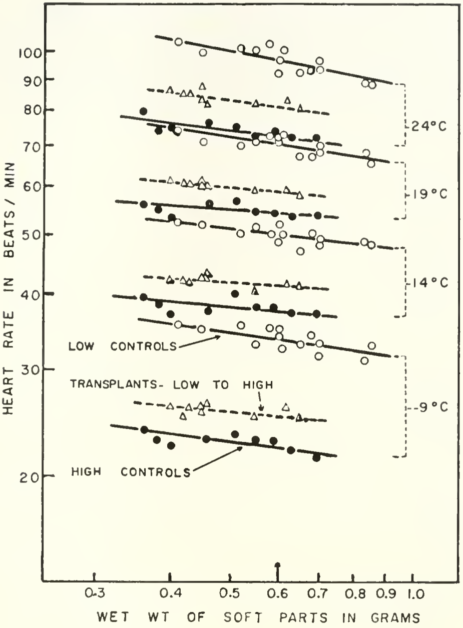

FIGURE 4. Relation between heart rate and wet weight of soft parts for second experimental transplantation. Duration of experiment 14 days. Each point represents one individual. Low-level controls measured at start of experimental period; high-level controls measured at end.

TABLE II

Calculation of per cent acclimation after transplantation from low- to high-level. Heart rate values are from equal weight (0.6 gm.) high- and low-level controls at start of experiment and high controls and transplants to high-level after 14 days. Rate values are taken from Figure 4. Per cent acclimation of transplants calculated from the difference in rate of low- and high-level controls at beginning and end of experiment. Performed in January 1954.

Temp. ° C.	Mean heart rates of average 0.6 gm. animals in beats/min.				% acclimation
	High controls 12/17/54	Low controls 12/17/54	Transpl. to high 1/4/54	High controls 1/4/54	
9	21.0	33.0	25.0	22.5	76.0
14	34.0	49.0	41.0	37.0	66.5
19	52.0	69.0	59.0	54.0	66.5
24	73.0	97.0	81.0	72.5	65.5
29	92.0	126.0	106.0	95.0	64.5

ning and end of the experimental period, we can roughly compare the response after 14 days with that after 29 days (Table II). At the 5 temperatures shown, an average of 68% of the difference in heart frequency between the groups has been achieved by the transplants in 14 days. However, since the transplants are acclimating to a temperature lower than that of the high-level habitat (see section on behavioral response), 68% may be too low a figure.

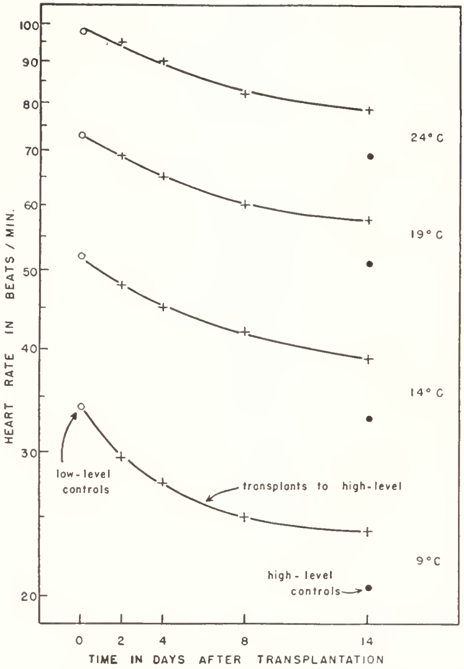

FIGURE 5. Acclimation of heart rate in low-level transplants to high-level 2, 4, 8, and 14 days after transplantation. Points represent equal weight (0.6 gm.) animals taken from the weight regression curve of each sample. Low-level controls measured at beginning of experiment, high-level controls at end and low-level transplants to high-level at days indicated.

It is of interest to notice that there is a proportionately greater change in heart frequency of the transplants at 9° C. than at any of the higher temperatures up to 29° C. This differential response may have as a basis the difference in slope of the rate/temperature curves of high- and low-level controls. The Q_{10}'s of the heart rate show the low-level or cold acclimated group to be less sensitive to temperature change at lower temperatures, *i.e.*, the curve is flatter between 9° and 14° C. An average 0.6-gm. animal from low-level has a Q_{10} of 2.33, while that of equal weight

TABLE III

Calculation of per cent acclimation 14 days after transplantation from low- to high-level and calculation of Q_{10} at several temperatures, 2, 4, 8, 14 days after transplantation. Heart rates obtained as in Table II. Per cent acclimation calculated as in Table II. Performed February 1954.

Temp. ° C.	Heart rates of average 0.6 gm. animals in beats/min.							Acclimation after 14 days %
	High cont. 2/1/54	Low cont. 2/1/54	Trans. to high. Days after trans.				High cont. 2/15/54	
			2	4	8	14		
9	20.0	34.0	29.5	27.5	25.0	24.0	20.5	74.0
14	32.0	52.0	48.0	45.0	42.0	39.0	33.0	68.5
19	51.0	73.0	69.0	65.0	60.0	57.5	51.0	70.5
24	67.0	98.0	95.0	90.0	82.0	78.0	69.0	67.5
Q_{10} values from these figures								
9–14	2.56	2.34	2.65	2.68	2.80	2.64	2.67	
14–19	2.54	1.97	2.07	2.08	2.04	2.17	2.38	
19–24	1.77	1.80	1.90	1.92	1.87	1.86	1.83	

high-level and transplant to high-level is 2.70 and 2.69, respectively, after 14 days. The difference in Q_{10} decreases with increasing temperature. The change in slope of the rate/temperature curve requires a proportionately greater change in heart frequency at lower temperatures.

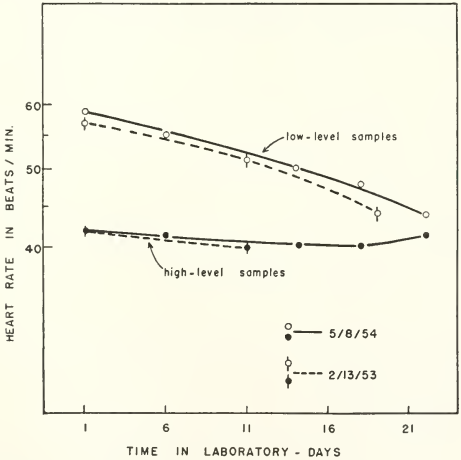

FIGURE 6. Heart rate during laboratory acclimation. High- and low-level samples kept cool (14° C.) and without food. Samples taken on days indicated; heart rate recorded at acclimation temperature (14° C.). Points represent equal weight (0.5 gm.) "average" animals taken from weight regression curves.

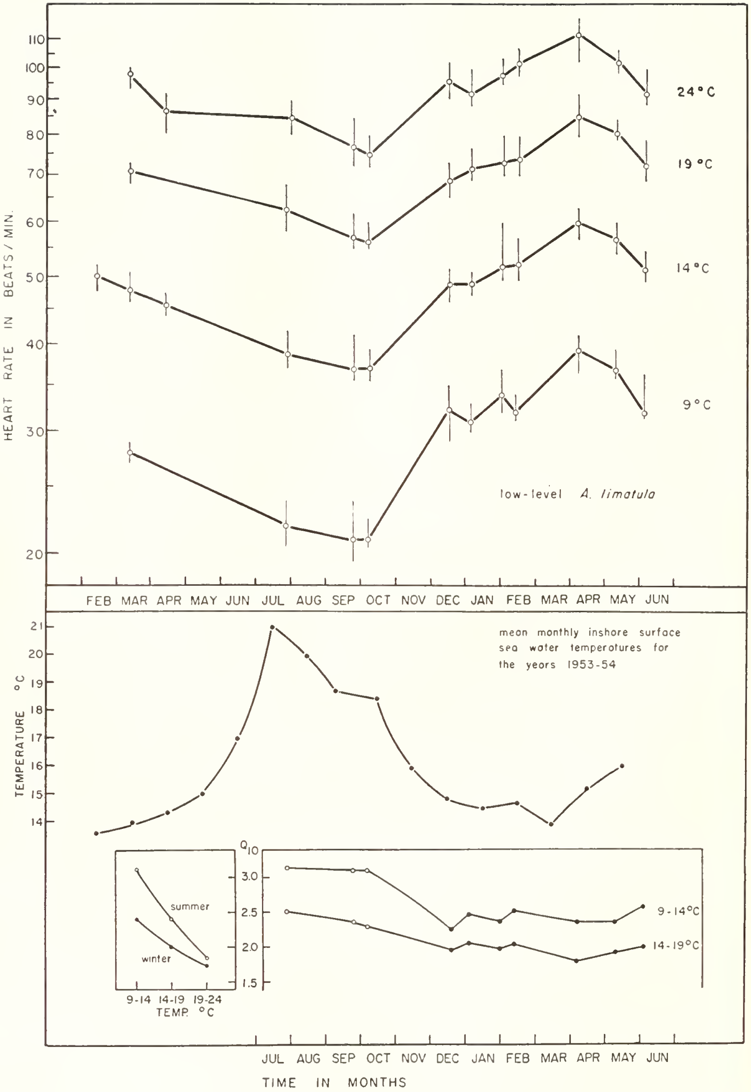
HEART RATE IN BEATS / MIN.
110
100
90
80
70
60
50
40
30
20
24 °C
19 °C
14 °C
9 °C
low-level A. limatula
FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC JAN FEB MAR APR MAY JUN
TEMPERATURE °C
21
20
19
18
17
16
15
14
mean monthly inshore surface sea water temperatures for the years 1953-54
Q_{10}
3.0
2.5
2.0
1.5
summer
winter
9-14 14-19 19-24
TEMP. °C
9-14°C
14-19°C
JUL AUG SEP OCT NOV DEC JAN FEB MAR APR MAY JUN
TIME IN MONTHS

FIGURE 7.

Fourteen-days: time course. In early February, 1954, 100 low-level limpets were moved to high-level at site 4. A total of 60% were recovered. Of 50 low- and 50 high-level controls, 50% and 60%, respectively, were recovered. Transplants were collected 2, 4, 8, and 14 days after transplantation.

Within two days a decrease in the heart rate of transposed individuals is noticeable. The further decrease in heart rate is non-linear plotted semilogarithmically, declining most rapidly in the first 8 days (Fig. 5).

Roughly 70% of the difference in heart rate was achieved by 14 days (Table III). This figure is arrived at by using the high-level control values (at 14 days) as a criterion of complete acclimation. However, for the reason given above (see second experimental transplantation), 70% is too low a figure.

The response of the transplants to short term temperature steps is remarkably similar in both this and the previous experiment. Not only is there a proportionately greater decrease in heart frequency at 9° than at 24° C. after 14 days, but the differential response is apparent after two days. Table III shows the calculated Q_{10}'s of the heart rate for equal weight controls and transplants. The change in slope at the lower end of the rate/temperature curve is conspicuous within two days.

Behavioral Response to Transplantation

On all three occasions, when low-level limpets were transposed to the level of high-water individuals, it was observed that the transplants moved from the site of placement.

In the first group of low- to high-level transplants, recovered on April 11, 1953, 29 days after transplantation, all surviving animals were found buried beneath a shell and gravel deposit at depths to 6 inches. High-level controls were recovered from the basalt outcroppings where they were naturally located, whereas the transplants, originally placed in close proximity to the controls, had migrated vertically downward from 1–6 inches out of the direct sunlight and into the damp deposit. A return to their old level is considered to be impossible on the boulder strewn beach.

The identical behavioral response was elicited from transplants in subsequent experiments. All surviving animals were found to have moved either vertically downward or horizontally under overhanging rocks, in both cases into cooler and damper regions. During the closely watched experiment, migratory movements were found to be complete two days after placement. High-level individuals, again, remained where they had been placed, in some cases in the identical spot.

The behavioral response to transplantation has bearing upon the heart frequency relations and is discussed in connection with those measurements.

FIGURE 7. Heart rate as a function of season for low-level *A. limatula*. Upper four curves —horizontal lines connect points for equal weight (0.6 gm.) animals taken from weight regression curves. Vertical lines denote total variation around regression lines within the weight range of 0.4–0.8 gm. The weight selected for comparison is from the middle of the usual range of weight at any collection period and from the comparable linear segment of the regression curves. Fifth curve—mean monthly inshore surface water temperatures in °C. from Redondo Beach, California (area adjacent to northernmost collection site) for the years 1953–1954. Points are monthly means calculated from the values of four daily recordings made between 9:00 A.M. and 6:00 P.M. Lower two curves—Q_{10} values calculated from the data above (upper 4 curves) for the temperature intervals shown. Inset—Q_{10} plotted against temperature to compare summer and winter averages.

Laboratory Studies

Additional, though indirect, laboratory evidence substantiates the field studies on acclimation. High- and low-level *A. limatula* were maintained at 14° C. in the laboratory without food for periods up to 22 days. Five to 10 individuals were withdrawn, one day after collection and at intervals thereafter, and the heart beat counted at 14° C. The heart rate of an average weight low-level animal, selected from the weight regression curves, decreased 25% of the initial rate after 22 days. A similarly obtained equal weight animal from high-level showed no appreciable decrease in rate in the same number of days (Fig. 6).

At the time of collection, low-level animals were living in the field at approximately the temperature of the experiment. The decrease in heart rate is presumed to be due to starvation uncomplicated by a tendency to acclimate. High-level animals, on the other hand, were living in the laboratory at a temperature lower than that in the field. At 14° C. two opposing forces are at work on these animals: starvation tending to decrease the heart rate and acclimation to a lower temperature tending to increase it.

Effect of Season on Heart Rate

Low-level population. Low-level *A. limatula*, collected during the winter and spring, have faster heart rates at all temperatures from 4° to 29° C. than animals of equal weight collected in summer (Fig. 7). The seasonal trend in heart frequency is in good agreement with the change in the mean monthly surface water temperature. In general, there is an inverse relationship such that an increase in temperature is followed by a decrease in heart rate which increases again with waning temperatures. The difference in winter and spring recordings of 1953 and 1954 has a basis in the habitat differences of the samples taken during those periods. Animals were collected from different sites during the comparable periods of each year. Low-level animals collected in 1953 were apparently slightly more subject to warming than were those animals collected in 1954.

The mean absolute change in rate (from winter to summer) at 24° C. is greater than at lower temperatures but the proportionate change at 9° C. exceeds that at higher temperatures. At 9° C. there is a maximum 46% change in rate as compared with 29% at 24° C.

Temperature sensitivity of the heart rate (as measured by the Q_{10}) similarly changes with season (see Fig. 7 and Table I). Winter and spring animals show lower Q_{10}'s and thus decreased sensitivity to temperature change between 9° and 24° C. With increasing temperature the difference in Q_{10} between winter and summer animals decreases. There is no appreciable difference if we compare Q_{10}'s at temperatures at which winter and summer animals show equal rates (at 14° C. or above for winter animals).

High-level population. When the seasonal change in heart rate of high- level forms is examined, the picture is less clear than that obtained for low-level forms (Fig. 8). There is a suggestion of the inverse relationship with seasonal temperature change, but the range in heart frequency is smaller and a response to short term temperature fluctuations, of the order of several days to a week, is evident (see February, 1954, Fig. 8). Although high-level animals were always chosen from the upper extreme of the intertidal range of the species, the possibility of habitat differences between samples cannot be ruled out.

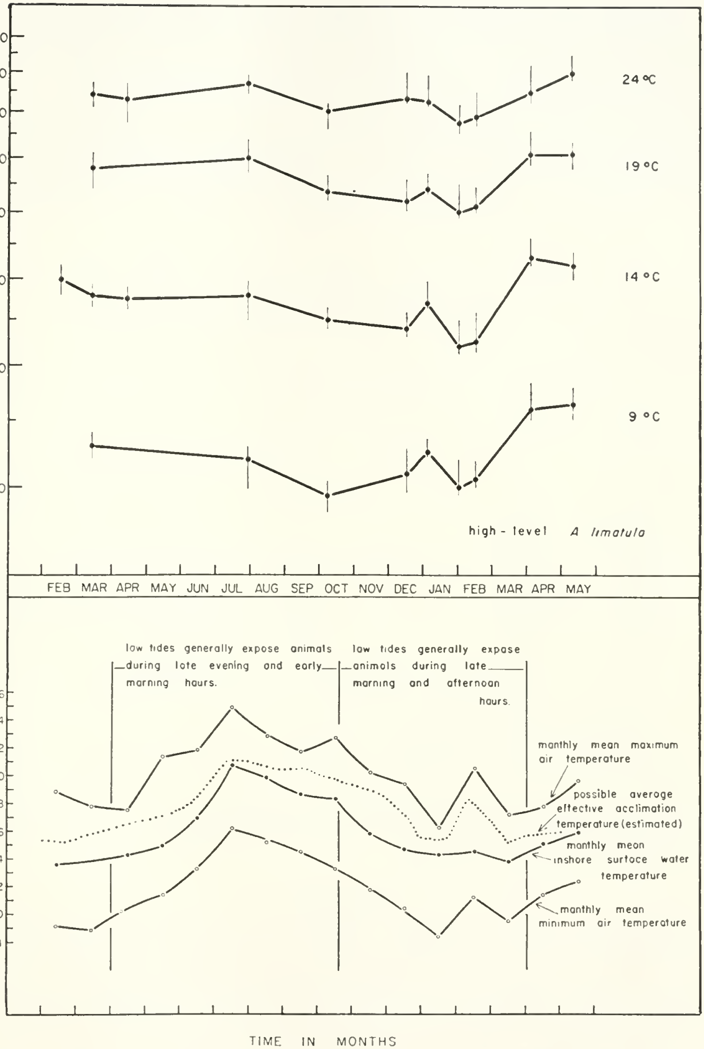

FIGURE 8. Heart rate as a function of season for high-level *A. limatula*. Parameters of upper 4 curves are the same as in Figure 7. Lower series of curves show (1) mean maximum and minimum monthly air temperatures (—○—) taken 4 times daily at Santa Monica, California; (2) monthly mean inshore surface water temperatures (—●—) as in Figure 7 but on reduced scale; and (3) possible average effective acclimation temperature (estimated) based on the heart rate response (· · · · ·).

High-level animals begin pre-spawning buildup of gonadal tissue in July and appear to maintain ripe gonads through October (see section on gonad size). The rather rapid heart rates in late July and possibly also in early October may be due to an increased metabolic activity associated with gametogenesis.

Table I shows the Q_{10} values calculated from the curves in Figure 8. Samples taken during "characteristic" winter months (January, April) show decreased temperature sensitivity (lower Q_{10}'s) than samples taken in "characteristic" summer months (August). However there is a marked lack of consistency in the temperature sensitivity of the heart rates of high-level animals. Samples taken during months of unseasonal temperature fluctuations (February, 1954) do not show the Q_{10} associated with adaptation of the heart rate to a warmer temperature, although the rates themselves have adapted.

Table IV

Gonad size as a function of season in high- and low-level A. limatula

Date of collection	Average gonad size in % wet weight of soft parts					
	High level	Gonads turgid	No. of specimens	Low level	Gonads turgid	No. of specimens
2/13/53	8.6		20	29.3		26
3/13/53	7.6		14	16.3		12
4/11/53	0.0		22	16.1		21
7/12/53	—	X	18	—	X	18
7/27/53	21.8	X	14	20.2*		7
9/25/53	28.1†	X	6	—**		12
10/ 7/53	29.4		12	17.1		15
12/17/53	6.6		13	21.7		19
1/ 4/54	7.6		20	31.1		12
2/ 1/54	0.0		16	20.4		15
2/15/54	0.0		12	15.9		10
4/ 6/54	0.0		14	17.0		18
5/ 8/54	0.0		8	15.6		11
6/12/54	15.6		14	16.1		12

* Six additional animals spawned in field—weights not taken.

** All animals spawned in field—weights not taken.

† Five additional animals spawned in laboratory—weights not taken.

Gonad Size

In relation to intertidal height. Gonad size, as per cent of the wet weight of soft parts, has not been found to vary systematically with the size of the organism over the range of 0.3–1.2 gm. Therefore, the mean gonad weight, as per cent of the body weight, of the animals of each collection period was calculated. These values are presented in Table IV.

Excluding the period roughly from early June to mid-October, low-level *A. limatula* appeared to maintain a larger gonad than high-level forms of the species. Mean gonad weight of low-level individuals did not fall below 15% of the body weight during the 1½-year period of observation. High-level individuals, from approximately November until June, possessed either small or negligible amounts of gonadal tissue with as many as 60% showing indeterminate sex. Indeterminate sex among low-level individuals was observed during the month of September after spawning occurred in the field.

In relation to season. Natural spawning among high- and low-level individuals does not appear to occur at similar times. Turgidity of the gonad (regardless of size) and deformation of the female gametes have been used to indicate the presence of pre-spawning ripeness—in agreement with Fritchman (1953). In July, samples from both high- and low-level revealed this condition (see Table IV). Later in the same month, partial or complete spawning of 50% of the low-level sample had occurred. Spawning of the entire low-level population is assumed to have taken place before late September.

Table V

Effect of transplantation on size of gonad in A. limatula

Date of collection	Average gonad size in % wet weight of soft parts							
	Controls				Transplants			
	High level	No.	Low level	No.	Low to high	No.	High to low	No.
Experiment I—29 days								
3/13/53	7.6	14	16.3	12				
4/11/53	0.0	22	16.1	21	0.0	18	15.9	20
Experiment II—14 days								
12/17/53	6.6	13	21.7	19				
1/ 4/54	7.6	20	31.1	13	21.9	10		
Experiment III—at intervals to 14 days								
2/ 1/54	0.0	16	20.4	15				
2/ 3/54					19.9	9		
2/ 5/54					21.4	9		
2/ 9/54					21.2*	4		
2/15/54	0.0	12	15.9	10	7.3**	4		

* Six other animals spawned in laboratory.

** Six other animals without weighable gonads at time of collection.

High-level individuals, on the other hand, showed a persistent turgidity of the gonads throughout late September in the field, although under maintained temperature (17°) in the laboratory, 50% of the September sample spawned. In early October, weight of the gonads of high-level forms had not significantly changed, but turgidity was no longer apparent. By December, 50% of the high-level sample was devoid of weighable gonadal tissue; by February, 100% of the animals showed this condition and remained so until the following June. After September, spawning among the low-level population was apparently at an end; the gonads then returned to approximately the average weight for non-spawning months. However, a second buildup of the gonads occurred in midwinter (January and February of two consecutive years) which, though of substantial weight, did not show the characteristic pre-spawning turgidity.

Effect of transplantation. Gonads of transposed animals were examined subsequent to recording of the heart rate. Data are available from three experimental transplantations and have been summarized in Table V.

1. After 29 days, in the spring, a complete reversal of the gonad size was obtained. High-level transplants to low-level developed gonads whose average size was not significantly different from that of the low-level controls.

2. During January, 1954, the second gonadal buildup occurred among low-level individuals. While the average gonadal weight of low-level controls increased by aproximately 50% in 14 days, that of the transplants from low- to high-level did not increase; rather they averaged the same size as before transplantation.

3. During February, 1954, animals were collected 2, 4, 8, and 14 days after transplantation from low- to high-level. No change was observed in the size of the gonads up to 8 days after transplantation although 60% of the individuals from the eighth day of collection spawned in the laboratory (19° C.). At the end of 14 days, 60% of the transplanted individuals possessed negligible gonads and the weights of the remaining 40% averaged less than one-half that of the low-level controls on the same date.

DISCUSSION

Microgeographic variation. The preceding data show that the microgeographic intertidal distribution of a gastropod, *Acmaea limatula*, is reflected in certain physiological and morphological differences (see Segal, Rao and James, 1953, preliminary report). The differences found in relation to shell and body size have been reported previously (Segal, 1956). High intertidal *A. limatula* are found about one meter above zero datum; low intertidal *A. limatula* are found at zero datum and below. High-level forms show a slower heart rate than low-level forms when both are measured at any given temperature from 7° to 29° C. Comparisons show that high intertidal animals are exposed about 50% of the time and are subjected to air temperatures which rise above and fall below that of the ocean. Low intertidal animals are submerged over 90% of the time and live essentially at the temperature of the ocean.

We do not have the complete curve of temperature against time for the high-level animals; therefore, we do not know how the temperatures they are acclimated to are related to the temperature fluctuations they are subjected to. We only know that these animals respond as if they are living at a higher temperature than that of the ocean. In this regard, Kirberger (1953) maintained an annelid, *Lumbriculus variegatus*, for 12 hours alternately at 16° and 23° C. for 8 to 14 days. She compared the O_2 consumption of these animals with that of two groups kept solely at 16° and at 23° C. for the same period of time. Those kept at the alternating temperatures averaged the fluctuations and responded as if they were adapted to 19° C. Animals maintained at the constant temperatures showed the typical compensatory response, *i.e.,* those animals from 16° C. consumed more O_2 than those from 23° C. when measured at the same temperatures.

Numerous studies have shown similar physiological differences to exist between macrogeographically distributed populations of a species. The question has been raised as to whether these animals are, in fact, members of the same species. Such latitudinal studies where the physiological differences are clearly correlated with habitat temperatures (Mayer, 1914; Spärck, 1936; Fox and Wingfield, 1937; Fox, 1939; Roberts, 1952; Dehnel, 1955) are sufficiently similar to intertidal microgeographic studies to warrant the suggestion that the same compensatory phenomenon is involved. High intertidal individuals, similar to warm seas populations, behave as though they are warm-adapted relative to low intertidal individuals and cold seas populations. Rao (1953), and Segal, Rao and James (1953), in the only studies where microgeographic and macrogeographic physiological differences have been compared in the same species (*Mytilus californianus*), show that 2½ feet of vertical separation is equivalent to about 350 miles in latitudinal separation. The rate of water propulsion in low-level northern mussels differs as much from that in low-level southern mussels as the rate in low-level southern mussels differs from that in high-level southern mussels 2½ feet higher in the intertidal zone. The data suggest that we are dealing with the phenomenon of individual adjustment to habitat temperatures; in short we may hypothesize that this is a phenotypic adaptation. In the present study we have made a more direct test of this hypothesis.

If a physiological rate character is, in time, readily reversible under changed temperature conditions, we may say that this rate attribute is acquired during the ontogeny of the individual. These changed temperature conditions may be artificially imposed by laboratory acclimation or by transplantation of the organism in the field; they may be naturally imposed by the changing season.

When low-level *A. limatula* were transposed to high-level, slowing of the heart rate was evident in two days and full adaptation was accomplished within 14 to 29 days. The reverse, adaptation to cold, was also complete within 14 to 29 days (Figs. 3, 4, and 5). Thus, the difference in heart rate of individuals at different intertidal levels was shown to be reversible under habitat conditions.

There are few published reports on transplanting individuals of a species from one habitat to another, using some physiological rate character as a measure of adjustment. Sumner and Lanham (1942) and Loosanoff and Nomejko (1951) report instances of transplantation with no apparent acclimation. These results may be due either to the inability of a homogeneous species to acclimate as in the first reference cited, or to the existence of true physiological races as in the second reference. Physiological races have been demonstrated previously both among field and laboratory populations (Brown, 1929; Goldschmidt, 1932, 1934; Hovanitz, 1947; Stauber, 1950). The transplantation method seems to be effective for revealing the nature of intraspecific physiological and morphological differentiation (Moore, 1934; Segal, 1956).

Acclimation of the heart rate has also been shown to occur with the seasonal change in temperature. Low-level animals have about the same rate in winter and in summer at their respective field temperatures (Fig. 7). High-level animals show responses to unseasonal air temperature fluctuations which tend to mask the seasonal acclimation (Fig. 8). In substantiation of the field studies, acclimation to cold has also been demonstrated in the laboratory (Fig. 6).

Temperature sensitivity. The sensitivity of the heart rate to temperature change, measured by Q_{10}, has also been shown to vary with intertidal height. Between 9° and 19° C., but not as clearly above 19° C., low-level, cold adapted organisms show lower Q_{10}'s than equal weight high-level organisms (the rate of change over temperature *intervals* is used rather than at temperature points because it is believed that rates of 1° C. increments are necessary for a reliable estimation of the change in rate at a given temperature). Bělehrádek (1935), using examples taken from data of various investigators, points out that temperature coefficients commonly increase with the adaptation of the protoplasm to higher temperatures. This thesis is further strengthened through additional evidence of Rao (1953) and recalculations by Rao and Bullock (1954) of earlier equivocal data.

Of interest are the temperature relations of high and low intertidal groups. Although low-level animals respond as though they are cold acclimated relative to high-level animals, environmental temperatures below 13° C. are probably rarely encountered in this area. High-level animals do meet with such temperatures during the winter and spring months when the higher of the two low tides and the lower of the two high tides of each day are of insufficient magnitude to cover the animals. It is worthy of note that on the two occasions when heart rates were measured at 7° C. (at 4° C. both groups show cold depression and cessation of beat in a fair percentage of each sample), low-level animals show higher Q_{10}'s between 7° and 9° C. It indicates that the low-level, cold acclimated group is paradoxically approaching cold depression at a higher temperature than the relatively warm acclimated, high-level group. It further suggests that the physiological temperature range (that range of temperatures over which there is no observable indication of depression) extends farther into the cold in the warm acclimated group. Above 29° C. heart beats could no longer be counted with accuracy, but the very fact that high-level animals have been found with higher body temperatures in the field, while the surface ocean temperature rarely if ever has exceeded 24° C. in this locale, permits the interpretation that the physiological range similarly extends farther into the warm. Dehnel (1955) reaches the same conclusion for optimal temperature range of larval growth within the species in populations from Southern California and Alaskan waters.

Additional confirmation exists for the thesis that cold-adapted organisms show lower Q_{10}'s and thus greater independence to temperature change. Winter animals as compared with summer animals have lower Q_{10}'s, at least from 9° to 24° C., and animals transplanted from low-level (cold) to high-level (warm) show an increased temperature dependence (higher Q_{10}'s) within two days.

Scholander *et al.* (1953), in a metabolic study of arctic and tropic poikilotherms, suggests that a low Q_{10} would only be advantageous to offset the effects of changes in temperature due to diurnal, seasonal, or migratory factors. The authors state that no such adaptation was found among the species which would profit from a low Q_{10}: temperate water forms, fresh water forms, and terrestrial forms. Rao and Bullock (1954) agree that at present no general case can be made for lower Q_{10}'s in forms exposed to changing temperature but argue that cold adapted, *e.g.*, arctic species as compared with tropic species, do show lower Q_{10}'s even though they may not be normally exposed to changes in temperature.

The present study is unique in that comparisons are made between animals which are living under fluctuating temperature conditions (high-level, exposed) and animals which are living under relatively constant temperature conditions (low-level, submerged). In this locale the high-level forms are exposed to considerably higher temperatures than that of the ocean and these animals act as though warm adapted. As shown, the warm adapted animals have the higher Q_{10} in spite of the fluctuating temperature of the habitat. Similarly, the summer forms from both high- and low-level have higher Q_{10}'s.

These differences could not have been expected simply from measurements of the Q_{10} of the *species* at any one time. If individuals of a species residing in different microhabitats and from one season to another show variations in temperature sensitivity, a great burden is placed upon comparisons between species. Other than for species living in arctic and tropical seas, with their almost constant temperatures, it is doubtful whether Q_{10} values (or any temperature coefficient describing sensitivity to changes in temperature) of a physiological rate activity are meaningful except in very limited comparisons. The thermal history of one segment of a species is not the thermal history of that species. The range of Q_{10} values (at a given temperature) permissible within the genetic makeup of a species would describe the temperature sensitivity of that species.

Spawning and gonad size. If the data on spawning represent normal behavior, then low-level *A. limatula* spawn before high-level *A. limatula.* Now the question remains as to whether high-level *A. limatula* actually spawn. All low-level animals show partial or complete spawning by late August. High-level animals, on the other hand, if they do spawn, do so sometime between October and December. We are not sure that high-level animals spawn because gonadal turgor and deformation of the female gametes, which Fritchman (1953) considers as indicative of pre-spawning ripeness, were not present in October although the gonads of high-level animals were of large size. Therefore, we must assume either that (1) high-level animals do not spawn in the field, or (2) pre-spawning ripeness is not a necessary condition. Again, if spawning occurs, it is out of phase with that of the low-level population. Yet, Fritchman (1953), working with high- and low-level members of two species of the same genus in central California (*A. fenestrata cribraria and A. testudinalis scutum*), did not find a difference in spawning time.

With the warming of the ocean in May and June (Fig. 8), gametogenesis is stimulated in both high- and low-level populations. By July, all animals showed the gonadal turgor and deformation of the female gametes associated with the pre-spawning ripe condition. Spawning occurs in the low-level population; this population is submerged and therefore subjected to the more constant temperatures of the ocean. High-level animals, which are only submerged 50% of the time, do not spawn during this period of warmest average sea water temperatures (July, August, September: 21°–19° C.). But, by October, the characteristics associated with pre-spawning ripeness have disappeared although the gonads are still large. From October to December, when the average inshore surface water temperatures have fallen to 17° C. and below, the high-level animals lose their gonads. The trigger mechanism necessary to initiate spawning may well be a required time interval spent at a given temperature rather than the reaching or exceeding of that temperature for a short interval of time. Fifty per cent of the high-level animals (September collection; mean ocean temperature 19° C.) spawned in the laboratory after three days at 17° C.

The loss of the gonad sometime between October and December coincides with the seasonal tidal change; from October through April high-level animals are exposed during the late morning and afternoon hours (Fig. 8). It is during these hours that these animals are subjected to direct solar radiation and to heat conduction from the exposed rock substratum. The presence of a large gonad, or for that matter any gonadal material, would decrease the area under the shell available for water and thereby decrease the animals' ability to avoid desiccation (Segal, 1956). Low-level animals transplanted to high-level lost the gonadal material within two weeks (Table V). The evidence suggests that we are dealing with a non-breeding population living at the extreme of the intertidal distribution of the species.

SUMMARY

1. Highest and lowest members of a eurytopic intertidal species, *A. limatula,* have been compared in the following: heart rate, gonad size, and spawning behavior.

2. Within the weight range of 0.4 to 1.0 gm., the heart rate varies inversely with increasing weight. The regression coefficients fall between − 0.042 and − 0.172; thus no single expression is available to describe fully the effect of weight on heart rate in this species.

3. Sex and size of gonad (as divorced from turgidity) have not been found to contribute to the variation in heart rate between samples.

4. Comparing equal weight animals, it is found that low intertidal individuals have faster heart rates than high intertidal individuals at any temperature from 4° to 29° C.

5. From data on field temperatures it is suggested that the significant parameter of the intertidal difference is temperature. High-level animals are subjected to considerable periods of warmer as well as to some periods of cooler temperatures than are low-level animals.

6. An attempt was made to characterize the difference in heart rate by: transplanting the animals in the field, following the seasonal changes, and maintaining samples of both populations in the laboratory at a cool temperature (14° C.) and without food.

7. When low-level animals are transplanted to high-level their heart rates slow so that within 29 days it is equal to that of the high-level animals when measured at any given temperature. The half-acclimation time was about 6 days. In the field, acclimation to cold was also shown to be complete within 29 days.

8. Comparisons of the heart rate during winter and summer showed that both high and low intertidal animals have faster rates in winter at any given temperature from 9° to 29° C. Acclimation to cold was also shown in the laboratory.

9. The above results lead to the interpretation that the microgeographic difference in heart rate is a phenotypic expression of a compensatory phenomenon operating to maintain approximately equal heart activity in spite of the habitat temperature differences. Latitudinal differences in physiological rate activities which

are clearly correlated with habitat temperature are sufficiently similar to the intertidal differences reported here to warrant the suggestion that the same phenomenon is involved.

10. Low-level and winter animals show a heart rate that is less dependent on temperature changes in the range from 9° to 19° C. This same response is not observed consistently above 19° C. While both high- and low-level animals appear to be approaching cold depression below 9° C., the low-level animals are cold depressed at a higher temperature than the relatively warm-adapted high-level animals; the low-level animals possess a higher Q_{10} in this range. It is suggested that the physiological temperature range of the warm-acclimated group extends both higher and lower than that of the cold-acclimated group. In the field, the Q_{10} of the heart rate changes within two days after the animals are transplanted.

11. The size of the gonad also varies with intertidal height. Low-level animals maintain a larger gonad during winter and spring than do high-level animals. Transplantation also reveals this difference to be reversible.

12. Analysis of spawning behavior (using turgidity as the criterion of pre-spawning readiness) presents the possibility that either (1) the two groups spawn a number of months out of phase with each other, or (2) high-level individuals do not contribute to the breeding population.

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MEMBRANE POTENTIAL AND RESISTANCE OF THE STARFISH EGG BEFORE AND AFTER FERTILIZATION[1]

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Unequal distribution of ions between the interior and exterior is characteristic of living cells. In many thus far studied the concentration of potassium is much higher in the interior while the concentrations of sodium and chloride are lower. This ionic asymmetry is associated with a potential difference across the plasma membrane which is approximately related to the relative concentration of potassium (Höber, 1945; Hodgkin, 1951) according to the Nernst equation:

$$E = \frac{RT}{nF} \ln \frac{(K^+)i}{(K^+)_0} \qquad \text{or at } 20^\circ \text{ C.,} \qquad E\,(\text{in mv.}) = -\,58 \log \frac{(K^+)i}{(K^+)_0}.$$

Membrane potentials have been recorded from many cells (Hodgkin, 1951; Grundfest, 1955), including some whose internal potassium concentration is known, by means of a fine, saline-filled, microcapillary (Gelfan, 1927, 1931; Ling and Gerard, 1949) inserted through the cell surface. The magnitude of this potential in different cells ranges from 50 to 100 mv., inside negative. This indicates an internal excess of potassium approximately 9 to 50 times the external concentration, and is in approximate accord with the observed values in specific cases in which potassium concentration has been determined.

Several investigators (Gelfan, 1931; Rothschild, 1938; Kamada and Kinosita, 1940) had, many years ago, reported that they could find no potential difference across the membrane of echinoderm eggs.[6] Interest in this problem has sharpened recently because of two new factors. In the first place a number of workers (Scheer,

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[6] In a brief report Taylor and Whitaker (1926) mention experiments on eggs of the sea urchin *Clypeaster rosaceus* showing a potential difference, inside negative, of about 1 mv., which would be very low in comparison with other kinds of cells that have been investigated.

Monroy, Santangelo and Riccobono, 1954; Furshpan, 1955; Kao, 1955) have independently made similar observations in several varieties of marine eggs, using modern recording equipment and stable KCl-filled (Kamada and Kinosita, 1940; Nastuk and Hodgkin, 1950) microcapillary electrodes. In the second place, convincing data have become available (Rothschild and Barnes, 1953) showing that at least for the eggs of the sea urchin *Paracentrotus lividus*, the potassium content in the aqueous phase of the egg is 21 times higher than in sea water, while the internal sodium and chloride concentrations are, respectively, about $\frac{1}{9}$ and $\frac{1}{7}$ of those in sea water. It would therefore seem likely that in the sea urchin egg a membrane potential of about 80 mv., inside negative, should be observed.

The persistent failure to find a membrane potential prompted a re-examination of this problem with certain technical refinements which provide definitive verification of the entry of a microelectrode into the cell, as well as measurements of the resistance and capacity of the membrane. Parallel experiments with microinjection (Tyler and Monroy, 1955) helped to elucidate and overcome difficulties encountered in attempts to pierce the cell membrane of echinoderm eggs. A potential difference was thereupon found to exist across the membrane of *Asterias* eggs. Its magnitude was found to be somewhat lower than would be expected on the basis of the high internal K^+, which was also determined in these experiments. As in other kinds of cells that have been investigated, the membrane potential difference changes reversibly on changing the external K^+ concentration.

Although, as will be shown below, it is unlikely that penetration by the microelectrode had been attained in earlier work, several observers (Rothschild, 1938; Scheer *et al.,* 1954; Furshpan, 1955) have reported that eggs could be fertilized while apparently impaled. Fertilization was also successful in the present experiments with the electrode truly inside the egg. The effects of fertilization on the potential and on the electrical constants of the membrane were therefore also studied.

Methods

Tyler and Monroy (1955) carried out experiments attempting microinjection of fluids into eggs of *Arbacia, Echinarachnius* and *Asterias.* A smaller number of experiments were performed in the present series in an effort to penetrate eggs of *Arbacia punctulata* with microelectrodes. Confirming the experience of Chambers, (Pandit and Chambers, 1932, and personal communication) in both cases it was found that piercing the surface is difficult. Microelectrodes or micropipettes which appear to have penetrated, in actuality only carry the membrane before them even to the extent of creating a tunnel (as Dan, 1943, has also observed) as the microcapillary travels through the diameter of the egg. This was clearly revealed in the microinjection experiments of Tyler and Monroy (1955), in which it was observed that the membrane could form a tight sleeve around the inserted pipette, the latter then appearing to be within the cytoplasm of the egg. However, injected fluid (KCl-NaCl solutions) would simply expand this sleeve and flow out into the surrounding medium rather than into the egg. Eggs of *Asterias forbesii* behaved similarly, as illustrated in Figure 1, but in view of their larger size (average diameter, 146 μ) these were chosen in preference to eggs of *Arbacia* for further investigation. Penetration of these eggs was accomplished by the technique of jarring the preparation by a light tap on the table. This sudden vibration was especially effective after the indented plasma membrane had formed a tight sleeve around the electrode (as in Figure 1e) and was allowed to remain in this condition for a short while. Figure 2 illustrates eggs with one or two electrodes that have penetrated into the cytoplasm.

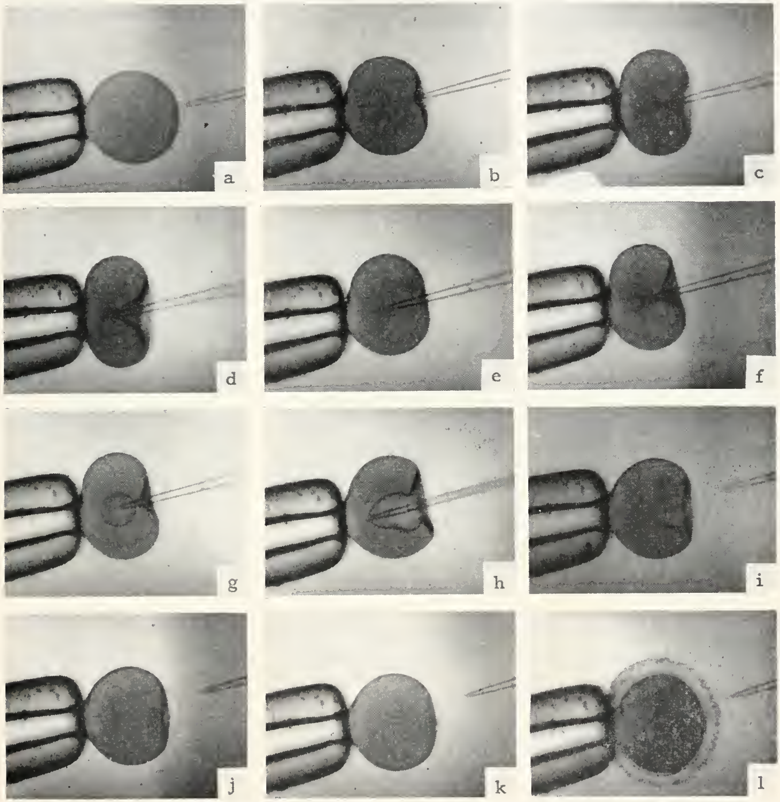

FIGURE 1. Photomicrographs showing apparent entry of a micropipette into an egg of *Asterias forbesii* held by a "sucking" pipette. Magnification, 104 ×. The micropipette, filled with isotonic NaCl–KCl solution containing chlorphenol red, is pushed through the vitelline membrane and indents the underlying surface (plasma membrane) forming a large conical depression (*d*). After about two minutes the depression closes over the pipette (*e*) and the latter appears to be within the cytoplasm. However, injection of fluid (*f* to *h*) shows that the walls of the depression had formed a tight sleeve around the shaft of the pipette. The fluid expands this sleeve and stretches the vitelline membrane, flowing out through the latter. Upon removal of the pipette (*i* to *k*) the remaining fluid is expelled as the egg rounds-up within some two minutes. The same egg with a fertilization membrane elevated at two minutes after fertilization is shown in *l*.

The experiments were carried out primarily on unfertilized eggs. A few measurements were made on eggs with germinal vesicles or fertilized prior to impalement by microelectrodes. The eggs were obtained from spontaneously shedding animals. They were kept at temperatures of 18 to 20° C. until used, one to five hours after shedding. The experiments were carried out mostly at about 25° C. and all the eggs used appeared to be normal. All those so tested, as well as parallel samples, were fertilizable. Some batches of eggs were stored at about 10° C. but these underwent spontaneous activation on transfer to the room temperature and were, therefore, not used in the experiments.

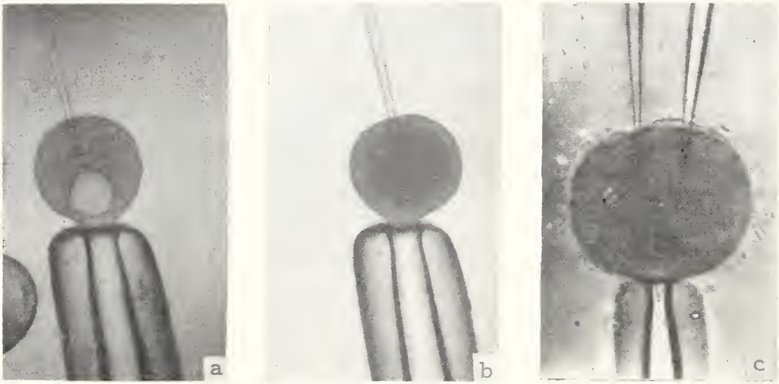

FIGURE 2. Photomicrographs of eggs of *Asterias forbesii* held on "sucker" and impaled on microelectrodes. Magnification: *a* and *b*, 116 ×; *c*, 200 ×. *a*, an egg with intact germinal vesicle; *b*, another after dissolution of the germinal vesicle; *c*, an egg fertilized after insertion of two microelectrodes. The tips of the microelectrodes are not visible.

All the impaled eggs, in the experiments involving fertilization, had undergone dissolution of the germinal vesicle, and were in various stages of the maturation divisions. Sperm were diluted in a solution of 10^{-3} molar Versene[7] in sea water, since the latter improves the fertilizing power of dilute sperm suspensions (Tyler, 1953). The sperm were introduced by means of a capillary pipette at a distance several millimeters from the egg. The time at which sperm were seen to approach the egg, as well as the time of formation of the fertilization membrane were noted for correlation with the measurements of the membrane potential.

Experimental arrangement

Sea water containing eggs was placed (Fig. 3A) on a transilluminated lucite plate mounted on a mechanical stage under a binocular microscope. The sea water was in continuity with one end of a sea water-filled tunnel, into the other end of which was inserted an Ag-AgCl reference electrode. One or two microelectrodes (tip diameters less than 0.5 μ), each individually carried in a micromanipulator, approached diagonally from above at a small angle. Opposite was another manipulator which held a glass suction pipette. This device (Tyler, 1955b), modified from the elastimeter of Mitchison and Swann (1954), was very useful for holding the egg fixed gently but firmly at one pole while the microelectrodes were pressed against the other (Figs. 1 and 2). The polished tip of the "sucker," somewhat smaller than the diameter of the egg, dipped into the sea water containing the eggs. The other end was flexibly coupled to a vertical glass tube which could be raised or lowered by a rack and pinion movement. The system was filled with sea water. By maneuvering the mechanical stage, any desired egg in the drop could be brought to the vicinity of the tip of the sucker. Lowering the upright created sufficient negative pressure to take up and hold the egg firmly against the tip. Manipulation was carried out under 80 or 160 × magnification.

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[7] Versene is the trade name (Bersworth Chemical Co.) of ethylene diamine tetraacetic acid.

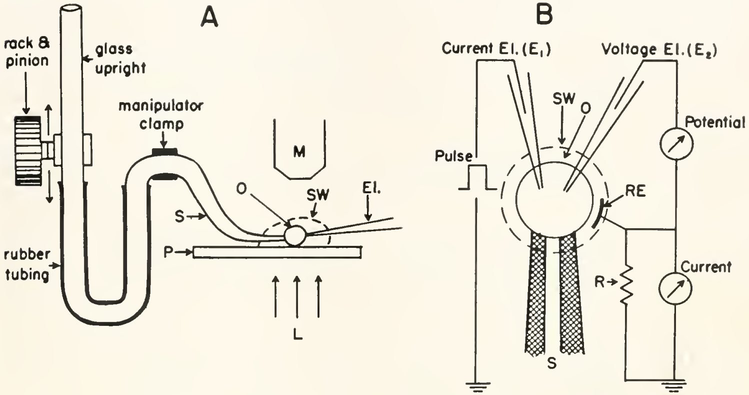

FIGURE 3. Diagrammatic illustration of the experimental arrangement. *A:* The mechanical and optical set-up. The egg (O) is shown (enlarged) lying in a drop of sea water (SW) on a lucite plate (P) which is illuminated from below (L) and observed through a microscope (M). The sucker (S) has a rack and pinion for raising or lowering the vertical tube. The electrodes (E) are inserted into the egg at the pole opposite that held in the sucker. *B:* The electrical arrangement. The two microelectrodes (El.$_1$ and El.$_2$) are shown in the egg (O). El.$_1$ is the current electrode fed through a pulse generator. El.$_2$ is connected to one grid of the potential recording amplifier. The other grid connects with the fluid (SW) as well as with a resistor (R) through the reference electrode (RE). Across R is the amplifier measuring the current in the pulse. When a single microelectrode (El.$_2$) was used, RE was grounded.

Electrical measurements

Determination of the membrane potential and the resistance and capacity of the membrane constituted the electrical measurements. For the former a single microelectrode, drawn prefilled with 3 *M* KCl (Kao, 1954), was sufficient. This was connected to a high impedance negative capacity input amplifier[8] and a cathode ray oscillograph. The external medium was grounded through the Ag-AgCl reference electrode, or in some cases the "sucker" was itself made the reference system. The standard sensitivity employed was 20 mv./cm. deflection on the face of the oscillograph tube so that changes in potential as low as two to three mv. could be detected. Visual observation was supplemented by photography of the trace.

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[8] Designed by Mr. E. Amatniek, electronic engineer at the Dept. of Neurology, Columbia University.

Determination of the electrical constants of the membrane required passage of a square pulse current through the membrane and the measurement of the potential difference created by this current across the resistance and capacity of the membrane (Fig. 3B). For this purpose two microelectrodes were inserted into the eggs (Fig. 2c). One of these was connected to a pulse generator delivering 12 or 30 msec. pulses of controllable amplitude, synchronized in rate and time with the sweep of the oscillograph. The external reference electrode was connected to a resistance, the other end of which was grounded, as was the return of the stimulator. An amplifier across the resistor recorded the IR drop in the latter and the current, I, through the membrane was calculated from this measurement. The sensitivity of the current trace on the oscillograph was 1 mv./cm. and with a 1 megohm resistor for the IR drop this amplitude of deflection corresponded to 0.001 μA.

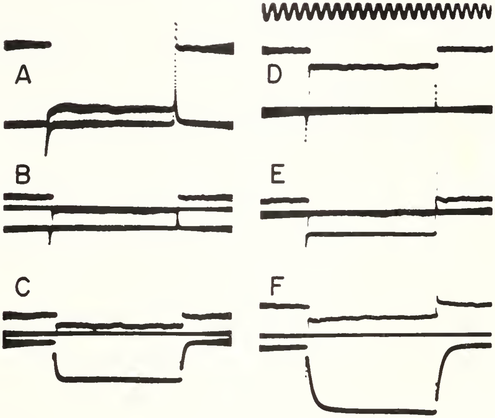

FIGURE 4. Current and voltage pulses as recorded before and after entry of two microelectrodes into the egg. *Left:* A large current pulse (*A*, upper trace) caused only transient capacitative artifacts in the voltage trace (below) as long as the electrodes were outside the egg. *B, C:* The current pulse was reduced. *B*, before and *C*, after penetration of the electrodes. A third trace, which represents the zero level at the time of entry, is seen at its correct position in *C*. The membrane potential, about −20 mv., has brought the voltage trace down. The square pulse of current is reflected in a membrane *IR* drop with retarded onset and decay. *Right:* Another egg. *D:* The current pulse caused only the capacitative artifact on the voltage trace. *E:* When the two electrodes were pressed against the egg, the voltage trace also recorded a deflection with rapid onset and decay. However, the steady potential was zero. *F:* A few seconds later, the electrodes had penetrated the egg, causing the characteristically slowed onset and decay of the voltage trace. A membrane potential of about −30 mv. is also seen. Time scale in msec., upper right.

The second microelectrode led, as before, to another amplifier, but in this case recording the membrane potential differentially, the second grid being connected to the indifferent electrode and the high end of the resistor. The potential change of this amplifier during the square pulse thus represented the *IR* drop across the membrane in series with the resistance of the fluid. Since the resistance of the latter was small compared with that of the membrane, it was neglected. From the knowledge of *I* obtained in the current record, *R* of the membrane could be computed. This was transformed to the specific membrane resistance R_M (ohm-cm.^{2}) by multiplying by the surface area of the egg (average diameter = 146 μ; surface = 6.7 × 10^{-4} cm.^{2}). The time constant (τ) when the rise and fall of the voltage trace (Fig. 4C, F) had reached 67% of the final value was measured from the records. The membrane capacity (C_M) was determined in μF/cm.^{2} from the relation $\tau = R_M C_M$.

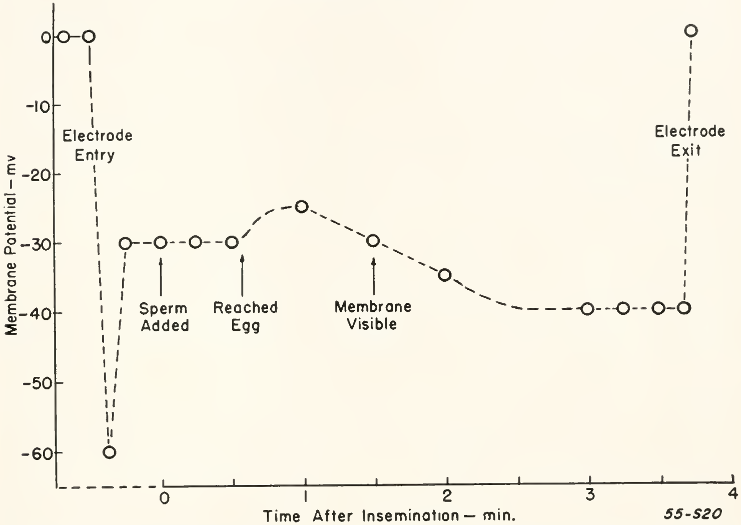

FIGURE 5. Membrane potential of Asterias egg and its changes on fertilization. Entry of the electrode into the egg caused sudden appearance of − 60 mv. membrane potential, which decreased to − 30 mv. rapidly. The time scale has its origin at the time sperm were added to the sea water. Within 30″ sperm were seen to have contacted the egg and at this time the previously steady potential decreased by 5 mv. Subsequently the potential again increased and remained steady at − 40 mv. until the electrode was removed. Absence of drift in the system is indicated by the return of the voltage trace to the initial value.

Certain precautions have to be taken in experiments of this type. The current electrode must be non-polarizable in the range of currents used for the measurements. This was checked at the start and end of each experiment. Secondly, applied current must be rather low. With a microelectrode tip of 0.5 μ diameter, 1 μA flow represents a current density at the tip of about 500 A/cm.^{2} which might lead to heating and perhaps breaking of the electrode. Furthermore, a flow of 1 μA through the whole surface area of the egg membrane is equivalent to a current of 1.5 mA/cm.[2] which is a high density, at least for the membranes of excitable tissues.

The use of the two microelectrodes and a current pulse served the additional important purpose of providing unequivocal evidence of the penetration of both electrodes into the egg (Fig. 4). When these electrodes were in the fluid the record of the voltage trace differed radically from the trace of the current pulse (Fig. 4A, B, D). The former showed only rapid short-lived deflections of opposite sign at the beginning and end of the applied pulse. These are attributable to capacitative coupling between the electrodes. During the major portion of the applied pulse, the voltage trace remained essentially at zero potential, reflecting the low resistance of the sea water and the consequently low *IR* drop across it. On occasion, when both electrodes were simultaneously pressed firmly against the egg, the voltage trace showed a large deflection which would be expected if the current path now included a high resistance formed by the surface of the egg membrane. However, this voltage record was characterized (Fig. 4E) by its faithful reproduction of the form of the current pulse, indicating that the recording electrode was not across

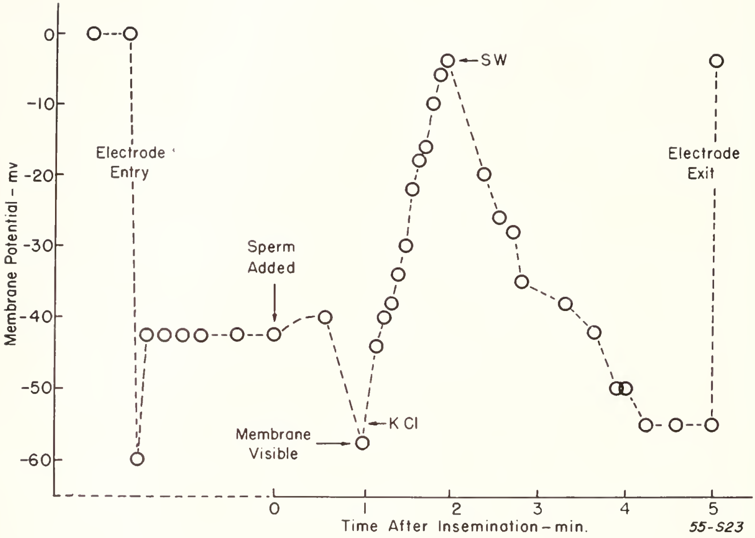

FIGURE 6. Depolarizing effect of externally applied KCl and its reversibility. The sequence up to one minute on the time scale is similar to that of Figure 5. When the membrane potential of the fertilized egg had reached its maximum value, the sea water was largely replaced with isotonic KCl. This caused rapid and almost complete depolarization, which was reversed on washing out the KCl with sea water. A drift in the amplifier of 4 mv. negative had changed the base line slightly.

the capacity of the membrane. When penetration occurred the voltage pulse changed in form characteristically, rising and falling more slowly than the current trace (Fig. 4C, F). This behavior, in view of our original uncertainty as to the existence of a potential difference across the egg membrane, proved valuable initially in definitely establishing the entry of the microelectrodes into the cytoplasm.

Ionic content of Asterias eggs

Measurements of the internal ionic milieu of *Asterias* eggs were not found in the literature. A sample of over two million eggs was therefore subjected to analysis by flame photometry. The procedure is detailed below in conjunction with the data obtained.

RESULTS

Membrane potential

In nearly all the eggs studied no potential accompanied apparent penetration by the electrode, but one or more taps on the table always caused the sudden appearance of a potential difference with the internal electrode negative, except in a number of eggs which cytolyzed as the electrode appeared to enter. The failure to obtain the potential initially was probably due to extensibility of the egg membrane. Jarring probably caused penetration of the membrane during the resultant vibration. The potential, upon penetration, reached values up to 60 mv., apparently instantaneously, then rapidly declined to a lower steady value (Figs. 5, 6). The steady value of the membrane potential ranged in different experiments from a low of 10 mv. to a high of 50 mv. (Table I), with the majority of eggs showing potentials of 20 to 30 mv.

TABLE I

Steady potential difference observed across the membrane of unfertilized eggs

P.D. in mv., inside negative	10	15	20	25	30	40	45	50
Number of eggs (Total 24)	3	2	5	1	8	3	1	1

In one additional egg, fertilized prior to penetration of the electrode, the membrane p.d. was initially −40 mv., and increased to −48 mv.

In one other egg only a p.d. of +10 mv. was obtained.

The larger initial value probably reflects more nearly the true potential difference momentarily disclosed as the fine tip of the microelectrode broke through the egg membrane. The subsequent release of tension of the latter, as it rounds up and moves farther onto the electrode, could create an imperfect seal of the membrane around the shaftlet and lead to partial short circuiting of the full potential. In most experiments the potential remained steady at the lower value as long as the electrode was left in the egg. Withdrawal was accompanied by an abrupt return of the oscillograph trace to the zero potential level. There seemed to be no consistent difference in the value of the steady potential if two electrodes were inserted simultaneously or sequentially. The number of experiments of this type was too small and the scatter of potentials too great to employ this method for calculating the possible magnitudes of leaks around the electrode.

Two eggs included in Table I, through which relatively high currents (0.5 to 1.0 μA) were later passed, cytolyzed in the course of the experiments. Five cytolyzed spontaneously, and two that had an intact germinal vesicle cytolyzed on subsequent penetration of this structure. In all cases cytolysis resulted in disappearance of the membrane potential. In three cases the potential disappeared with no observable cytolytic effects. Movement of the tip then again disclosed the membrane potential. These can be interpreted as eggs in which the cytoplasm had become sealed off from the electrode (Chambers, 1922) by a precipitation membrane (Heilbrunn, 1927, 1952; *cf.* Costello, 1932). In a few other experiments, all done with the same microelectrode, penetration was indicated at first by a small positive potential (about 10 mv.) which in all but one case reversed to negativity. The positive potential may have been due to increase of the electrode resistance by plugging of the tip as this pressed into the egg membrane. With the grid current of the amplifier about 10^{-11}A and positive, a shift of + 10 mv. would be caused by insertion of a resistance of 10^9 ohms. Another possibility is that the electrode had penetrated the egg, but had been sealed off from the cytoplasm while some leakage remained around the shaft. The internal negativity would then register as positivity on pickup by the external electrode.

Membrane potential upon fertilization

Eight eggs were fertilized while impaled. Insemination was done usually at least 5 minutes after impalement in order to ascertain that the membrane potential was steady and that the egg was not undergoing cytolytic changes. When spermatozoa were seen to have reached the impaled egg (about 15 to 30 seconds after insemination) the membrane potential suddenly decreased from its previously steady value. This change amounted to 5 to 10 mv., and was temporary (Figs. 5, 6). The membrane potential then began to increase gradually, eventually attaining a magnitude greater than the former steady value, and in some cases as large as that momentarily seen during entry of the electrode. This increased internal negativity persisted during the subsequent period of observation which, for the present series of experiments, was not longer than 5 minutes. The new steady value of potential, 5 to 20 mv. higher than before fertilization, was attained in 1 to 2.5 minutes, which was also the time at which the fertilization membrane had become distinctly elevated.

Ionic content of Asterias eggs

Estimate of the magnitude of the membrane potential to be expected requires knowledge of the ionic concentration in the egg. Determinations were therefore made of the K and Na content of *Asterias* eggs. The procedure was as follows:

A 100-ml. suspension of unfertilized eggs was prepared in sea water. From this, a one-ml. sample was removed by means of a wide-mouth (2.5 mm.) pipette. It was diluted 20-fold and a one-ml. portion used for counting the number of eggs. During these procedures precautions were taken to keep the suspension of eggs distributed as uniformly as possible. The final one-ml. diluted sample contained

1096 eggs. Therefore the 99 ml. of the original suspension contained 2.17 × 10^6 eggs. The eggs of the latter suspension were allowed to settle, and most of the fluid was drawn off. The remainder was then centrifuged for 15 minutes at 1500 × g, in graduated centrifuge tubes. The packed eggs measured 6.1 ml., and above them was an additional gelatinous, opalescent layer of 2.1 ml. representing the material of the gelatinous coat of the egg. Supernatant fluid was withdrawn to leave a total volume of 10 ml. of packed eggs, gelatinous layer and sea water. The eggs lost during the procedure were determined from counts of aliquots of the supernatants. Their number was 2128 or less than 0.1% of the total in the packed eggs. To the 10 ml. volume of packed eggs and supernatant, and separately to an equal volume of supernatant fluid, were added 10 ml. of sulphuric acid. The two preparations were allowed to stand overnight, transferred with distilled water washings to digestion flasks and boiled for about 4 hours, one ml. of 30% H_2O_2 being added to help clarify the material. Both preparations were then transferred to 100-ml. volumetric flasks and made up to that volume in distilled water. The original samples had thereby been diluted 10-fold. These were analyzed for K^+ and Na^+ by flame photometry.[9]

Table II

Measurements of diameters of eggs of Asterias forbesii

Number of eggs	1	3	1	1	2	2	1	3	Total: 14
Average of the two diameters (μ)	140	142.5	143.8	145	146.3	147.5	148.8	150	Average: 145.9

Table III

Determinations of K and Na content of eggs of Asterias forbesii

	Potassium	Sodium
(1) mM in 10 ml. of suspension containing 2.168 × 10^7 eggs	0.656	2.40 to 2.61
(2) mM in 10 ml. of supernatant	0.205	4.00 to 4.32
(3) mM in 6.47 ml. of supernatant	0.133	2.59 to 2.79
(4) mM in 3.53 ml. of eggs [(1)–(3)]	0.523	−0.19 to −0.18
(5) mM/ml. eggs	0.148	

To calculate the content of these ions in the eggs it was necessary to determine the egg volume, exclusive of interstitial fluid. The diameters of 14 eggs, in which the difference between diameters at right angles was less than 4%, were measured, with the results shown in Table II. The flame photometric determinations and calculated values of K and Na content are given in Table III.

On the basis of an average diameter of 146 μ the volume of each egg is 1.63 × 10^{-6} $cm.^3$ and that of the total number in the suspension is 3.53 ml. The latter value is 58% of the volume (6.1 ml.) of the packed eggs after the low speed centrifugation, and is in reasonable agreement with the value to be expected from the packing of spheres, plus a small allowance for adherent jelly coat. The 10-ml.

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[9] We are indebted to Dr. James Green of Rutgers University and to Dr. George Scott of Oberlin College for the analyses.

specimen containing eggs therefore was composed of 35.3% eggs and 64.7% interstitial fluid (including gelatinous coat material). From the measurements of the parallel sample of supernatant fluid the 6.47 ml. of the supernatant in the egg sample contained 0.133 mM K^+. The 3.53 ml. of eggs therefore contained 0.523 mM or 0.148 mM/ml. of eggs. This figure is about 15 times the K^+ concentration of sea water (0.01 mM/ml.). On the basis that the eggs contain approximately 75% water by weight and 80% by volume, the K^+ concentration becomes 0.185 mM/ml. of the water in the eggs. This corresponds fairly closely with the value of 0.210 mM/mg. of water found in eggs of the sea urchin *Paracentrotus lividus* (Rothschild and Barnes, 1953).

Table IV

Electrical constants of Asterias eggs

Expt. No.	Conditions	Steady membrane potential (mv.)	Maximum applied current (μA)		r_m (ohms)	R_M (ohm-cm.²)	τ (msec.)	C_M (μF/cm.²)
			Outward	Inward				
A. Maximum currents not exceeding .01 μA								
25	unfertilized	—*	.006	.006	4.0×10^6	2680	1.28	0.48
	fertilized	—	.006	.006	3.6	2410	1.25	0.52
26	unfertilized	−30	.006	.006	4.97	3330	1.92	0.58
27	unfertilized	−15	.005	.01	3.7	2430	1.04	0.43
28	unfertilized	−15	.005	.005	5.8	3880	2.75	0.71
	fertilized	−50	.005	.005	5.8	3880	1.6	0.41
B. Higher maximum currents								
7	unfertilized	−10	0.10	0.06	5.05×10^5	368		
8a	unfertilized	−10	0.10	—	11.	737		
b	unfertilized	−10	0.15	—	8.6	574		
11	unfertilized	−20	0.25	—	2.6	172		
6	unfertilized	−20	0.40	—	2.0	133		
13	unfertilized	−30	0.4	0.4	1.07	71.6		
1a	unfertilized	−30	1.0	1.0	2.6	174		
b	unfertilized		1.0	1.0	2.9	194		
14	unfertilized	—*	1.5	1.5	2.5×10^4	16.8		

* Potential not measured because of amplifier drift.

The supernatant contained about the same amount of sodium as does sea water, but the potassium concentration (0.02 mM/ml.) was twice that of sea water (Table II). For the calculation given above it was assumed that the extra K^+ derived from gelatinous material of the eggs, some of which remained in the supernatant. On the other hand, if this potassium had leaked out of the eggs during preparation for analysis, the initial concentration of the ion in the eggs would have been 0.168 mM/ml. of eggs and 0.210 mM/ml. of the water in the eggs.

Similar calculations for the Na content of the starfish eggs, using either the values of 2.40 and 4.00 mM/10 ml. of egg suspension and supernatant, respectively, or 2.61 and 4.32 gave a slightly negative value (− 0.2 mM per 3.53 ml. eggs) for this ion. In view of the high content of Na in sea water and therefore in the interstitial fluid of the egg suspension, the value for Na is much more sensitive to errors in determination of egg volume than is that of K. For example, if the actual egg-volume were 13% greater than determined, the calculated Na content would be zero while that of K would be 0.17 mM/ml. of the water in the eggs. We may therefore conclude that the Na concentration in *Asterias* eggs is less than one-twentieth, while the K concentration is between 17 and 21 times, the values found in sea water.

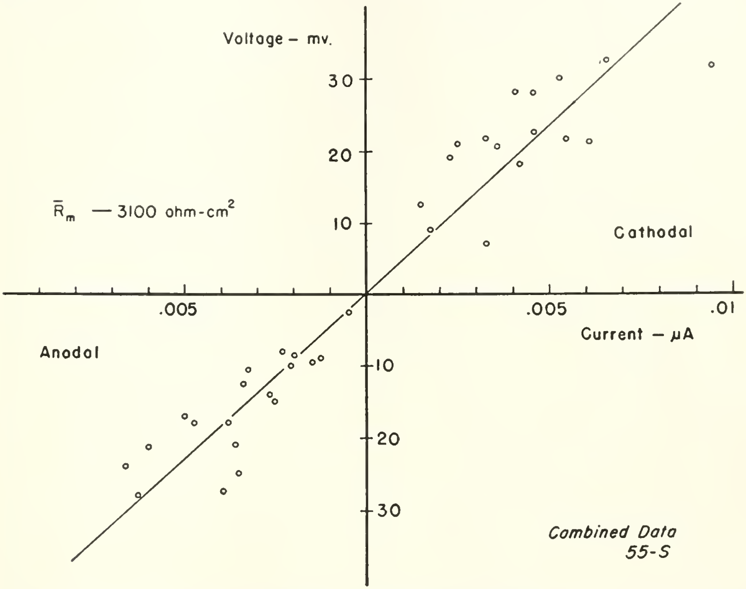

FIGURE 7. Membrane current-membrane voltage relation in four Asterias eggs. Two of the six experiments represent measurements done after fertilization. The slope of the straight line drawn through the combined data is the average resistance (r_m) and yields $R_M = 3100$ ohm-cm^2. *Anodal* signifies outward current and *cathodal,* inward.

Change in membrane potential on increasing external K^+

Four experiments served to test and demonstrate the sensitivity to K^+ of the potential difference across the egg membrane, but the relation between the external K^+ and the potential was not studied quantitatively. Within a few seconds after isosmotic KCl was added to the sea water surrounding an impaled egg the membrane potential decreased (Fig. 6) and reached almost complete depolarization. The K^+-rich solution was then replaced with sea water and the initial value of the membrane potential was again restored. Fertilized and unfertilized eggs responded in the same manner. The technical arrangement did not permit rapid washing out of the excess KCl and this probably accounts for the slower return of the initial membrane potential.

Membrane resistance and capacity

These electrical constants of the membrane were determined in 10 eggs with the square pulse technique described earlier. However, in six the maximum current densities were rather high (0.5 to 1.5 μA) and the results were probably affected by this condition. In six experiments with four eggs (two eggs were studied after fertilization as well as before) the maximum current of the square pulse was limited to 0.01 μA and these results are shown in Table IVA and Figures 7 and 8. The average membrane resistance was 3100 ohm-cm.[2] In the two eggs measured after the fertilization membrane was elevated there was no significant change in resistance (Fig. 8; Table IVA). In the six experiments employing high current densities (0.5 to 1.5 μA) the values of R_M were markedly smaller (Table IVB). No explanation will be attempted at present for this effect. Over the range of current densities used in all the experiments the relation between membrane voltage and current was linear. Assuming a specific resistance of 100 ohm-cm. (approximately

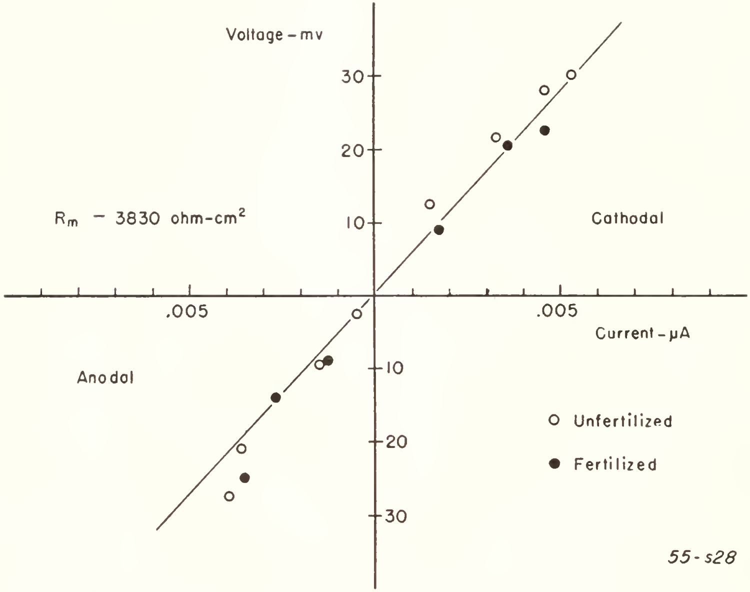

FIGURE 8. Membrane resistance before and after fertilization. The current-voltage relation of the same egg before and after fertilization.

three times that of sea water; Cole and Cole, 1936a, 1936b) for the cytoplasm, the droplet constituting the interior of the egg would have a resistance of about 8×10^3 ohms, surrounded by a membrane with a resistance several orders higher than this in magnitude.

Determination of the time constant (τ) and of the membrane capacity (C_M) was only approximate, because the oscillographic records of the membrane voltage change were made on too slow a time base. The average capacity (0.52 μF/cm.^{2}) is somewhat smaller than, but of the same order of magnitude as, the 1.1 μF/cm.^{2} obtained by Cole and Cole (1936a) with eggs of *Asterias forbesii*, and 0.7 to 2.7 μF/cm.^{2} listed by Cole and Curtis (1950) for unfertilized eggs of other marine animals.

Discussion

The original object of this investigation was to seek an explanation for the reported absence of a membrane potential in some echinoderm eggs. On the basis of Rothschild and Barnes' (1953) finding that sea urchin eggs contain 21-fold higher concentration of K^+ than does sea water it was to be expected that a membrane potential of about 80 mv. ought to be present. A membrane potential has now been found in *Asterias* eggs. Its magnitude, at least 60 mv. under what we believe to be the optimum condition, is smaller by about 15 to 20 mv. than might be expected on the basis of the values (17:1 to 21:1) that we obtained for the ratio of K^+ in *Asterias* eggs to that in sea water. A similar discrepancy is usually found in nerve and muscle fibers (Hodgkin, 1951: Grundfest, 1955).

The reversible depolarization of the egg membrane in response to increasing the external K^+ agrees with the behavior of other cells (Hodgkin, 1951), but the quantitative relation between potential and K^+ concentration was not tested in the present experiments. Another point of similarity relates to the low internal Na^+ concentration. *Asterias* eggs contain too little Na^+ for accurate measurement under the experimental conditions employed. Rothschild and Barnes (1953) found a concentration of 52 mM/kg. water in eggs of *Paracentrotus lividus* as compared with 485 for sea water, and the Na^+ concentration of various excitable cells is also considerably lower than in the fluid surrounding them (Hodgkins, 1951). It is thereby likely that an active transport mechanism exists in echinoderm eggs as it apparently does in other types of cells (*cf.* Brown and Danielli, 1954).

Rothschild (1938), and Kamada and Kinosita (1940) had considered, but rejected, the possibility that failure to obtain a membrane potential might be due to failure of electrode to penetrate the egg. Their decision was based on the apparent entrance of injection fluid into the egg. Furshpan (1955) believed that because in many experiments he had pushed the electrode clear through the egg, it must have been in the cytoplasm at some stage in the process and therefore considered his results to demonstrate absence of a membrane potential. However, as has been noted by Dan (1943) and by Tyler and Monroy (1955), the micropipette can readily tunnel through the egg, without entering the cytoplasm; the distended plasma membrane on one side simply joins that on the other and both are then perforated without injury to the egg which can later close the tunnel. It is pertinent to quote in this connection the remarks of Chambers (1922, p. 189):

> Pushing a pipette, especially a comparatively large one, into an egg cell frequently causes the surface of the cell to become invaginated and thus forms a deep pocket. The tip of the pipette, even if it should finally break through the surface, is apt to become separated from the protoplasm of the interior by the formation of a new surface film continuous with the original surface of the cell.

Chambers' conclusion was confirmed by Tyler and Monroy (1955, and illustrated in Fig. 1) and in the present experiments. It is now also well known, particularly from the work of Heilbrunn (1927, 1952; *cf.* Costello, 1932), that when the cytoplasm of eggs of marine animals, or of other cells, is brought in contact with Ca-containing solution a surface precipitation reaction occurs. The formation of such a precipitation membrane around the tip of the pipette might have been responsible for the lack of a membrane potential in the experiments of Rothschild (1938) in which injection tests indicated penetration. The absence of a potential in the experiments of Gelfan (1931), Kamada and Kinosita (1940) and Scheer *et al.* (1954) can also be attributed to failure of penetration or possible formation of a precipitation membrane.

A small positive potential was observed by Gelfan (1931) when the microelectrode presumably penetrated the germinal vesicle of the *Asterias* eggs. In our experiments, confirming Chambers (1921), puncture of the germinal vesicle invariably led to cytolysis of the egg. The sudden disappearance of the membrane potential when the germinal vesicle was impaled and cytolysis resulted, indeed served as additional verification of penetration in the experiments reported in the present paper. As noted, and discussed earlier, small positive potentials were occasionally observed in these experiments.

Although the absence of a membrane potential in echinoderm eggs reported by earlier observers is explained in the light of the present experiments, there remains the finding (Kao, 1955) that eggs of the killer minnow, *Fundulus,* do not exhibit a membrane potential. The precautions using two microelectrodes and an applied pulse, were also employed in those experiments to ascertain penetration of the egg membrane. However, the ionic composition of *Fundulus* eggs is unknown and the explanation for this different finding must remain in abeyance.

For the most part measurements of the membrane potential have been carried out on cells from tissue aggregates. Some data are, however, available for unicellular organisms. These are of interest not only because they provide a rather closer analog to eggs than do tissue components, but also because they, too, reflect the effects of improvements in technique. Telkes (1931) reported that amoebae have a membrane potential of 10 to 30 mv., inside negative. Buchthal and Péterfi (1937) found only small variable potentials of either sign (up to 3 mv.). Later, however, Wolfson (1943) succeeded in recording a membrane potential of up to 90 mv. in *Chaos chaos.* Dr. S. Crain, of the Department of Neurology, Columbia University (personal communication) has obtained similar values for the membrane potential in *Chaos chaos* and *Paramaecium.* As in eggs, it is difficult to penetrate the cell membrane and Wolfson used the device of applying negative pressure on the electrode, sucking the amoeba onto the shaftlet and eventually rupturing its cell membrane. Tauc (1953) found a membrane potential of 80 to 100 mv. in the plasmodium of a myxomycete. As in the *Asterias* egg, the high initial value decreased subsequent to penetration.

Effects of fertilization on the membrane potential

A second objective of these experiments was to examine whether or not changes in membrane potential accompany the events of fertilization. Péterfi and Rothschild (1935), using two small external electrodes placed on opposite sides of the surface of the frog egg, reported (p. 875) that "there are strong indications that the attachment of the spermatozoon to the egg results in an action potential being propagated over the egg surface, the action potential being characterized by having no recovery phase." Scheer *et al.* (1954), although they could not obtain a steady membrane potential with an electrode apparently inserted into eggs of *Paracentrotus lividus* and *Arbacia lixula,* reported transient potential differences upon fertilization. These changes consisted of a series of rapid pulses of irregular size, ranging from about 2 to 5 mv. They began at the time that the first visible reaction (cortical change) to the sperm was observed, persisted during the period of egg contraction and gradually disappeared. In the case of *Paracentrotus* eggs the pulses were much less frequent (often only one or two) than in *Arbacia* (as many as fifty). Scheer *et al.* (1954) point out that these changes are not strictly comparable to the action potentials of nerve and muscle. Furshpan (1955) saw no potential changes upon fertilization of eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus,* but as in the case of the other observers, neither was a steady membrane potential obtained with these eggs. Similarly Kamada and Kinosita (1940), using an "internal" electrode found no change upon fertilization, nor a "resting" potential in eggs of the sea urchin *Strongylocentrotus pulcherrimus.* An attempt to examine possible changes in membrane potential upon fertilization was also made recently by Allen, Lundberg and Runnström (1955) with external electrodes in contact through sea water in agar with the ends of a capillary tube in which a sea urchin egg was elongated and fertilized. They found no shift in potential but concluded that the technique proved inadequate for this problem.

The present series of experiments disclose that in eggs of *Asterias forbesii,* at least, there is a change in the membrane potential beginning with a sudden decrease when spermatozoa are seen to have made contact with the egg. The initial decrease of membrane potential is converted to an increase which reaches its maximum and steady value when the fertilization membrane is raised. These changes in membrane potential appear to develop smoothly without the occurrence of the pulses recorded by Scheer *et al.* (1954) with a capacitatively coupled amplifier.

The time course of the initial decrease in potential was not determined accurately. The optical system did not permit identification of the moment of sperm entry into the egg, and unfortunately photographic recording of the oscillograph traces was not done at sufficiently frequent intervals to define accurately the initial portion of the membrane potential change. However, visual observation at 1 per second sweeps, which was routinely done, indicated that the decrease took less than 10 seconds to reach its maximum value.

More detailed experiments will be required to establish whether or not the observed electrical changes in *Asterias* eggs are associated in time with the optical changes which accompany fertilization. The color change which passes over the surface of the sea urchin egg within a few seconds after the attachment of the sperm (Runnström, 1928) is a propagated response with a total conduction time of about 20 seconds at 18° C. (Rothschild and Swann, 1949). Birefringence of the surface disappears in about the same time (Monroy and Montalenti, 1947). These events therefore have about the same time course as the decrease and subsequent beginning of the return of the membrane potential to its former steady amplitude.

Studies on the rate at which block to polyspermy is established in sea urchin eggs (Rothschild and Swann, 1950, 1951, 1952; Rothschild, 1953, 1954) have led to the conclusion that there is a fast reaction which passes over the surface of the egg in about two seconds and that reduces the chances of refertilization by a factor of 20. This is followed by a slower change which in about 60 seconds reduces to zero the probability of a successful sperm-egg collision. The wave of explosion of the "Harvey-Moser granules" (Harvey, 1911, p. 523; Moser, 1939) takes about 15 seconds to traverse the surface of an echinoid egg from the point of attachment of the sperm (Endo, 1952). The time course of these events is about the same in the starfish egg judging from the fact that the fertilization membrane becomes visibly elevated at one to two minutes. As noted earlier, the new maximum of membrane potential is attained at about the time that the fertilization membrane is clearly visible.

A distinction must, however, be made in correlating the electrically and optically observed effects. The latter are initiated at the site of entry of the sperm and are slowly propagated from there. The former are recorded with an electrode inside a sphere of small diameter and this condition operates against the likelihood of observing discretely localized membrane potential change. If the electrical changes observed during fertilization have their basis in localized changes of the membrane, these are probably electronically averaged in the actual recording and would not reveal clearly a spreading electrical change which might accompany the propagated evolution of the optically observed phenomena.

The nature of the membrane events which lead to the electrical changes observed after fertilization can only be speculated upon. Potential change in excitable tissues is associated with change in membrane permeability and/or altered ionic flux (Bernstein, 1912; Hodgkin and Katz, 1949; Fatt and Katz, 1951; Hodgkin and Huxley, 1952; Eccles, Fatt and Koketsu, 1954). In the more complex bioelectric generators of frog skin or the gastric mucosa, changes in the transport respectively of Na^+ (Ussing, 1954; Kirschner, 1955) and Cl^- (Hogben, 1955) are involved. At least in the former case, metabolic activity and hormonal factors play an important role. Whether or not the electrical changes which occur upon fertilization are associated with known metabolic changes (*cf.* Brachet, 1947; Runnström, 1949; Tyler, 1955a) is not at present clear. Changes in ionic permeability such as might be indicated by the electrical data probably do occur. However, the time course of the K^+ accumulation is not known. Monroy-Oddo and Esposito (1951) have reported that sea urchin eggs gain K^+ upon fertilization, and such accumulation might account for the late increase in the membrane potential. Malm and Wachmeister (1950), on the other hand, report a slight decrease in potassium content, and a considerable increase in sodium, of sea urchin eggs upon fertilization. They attribute this to the fertilization membrane being permeable to the ions in the surrounding sea water, so that analyses after fertilization would show an increase in ions present in high concentration in sea water and an apparent decrease in ions like potassium previously accumulated by the egg. Shapiro and Davson (1941) had also reported no significant change in potassium content in sea urchin eggs upon fertilization. They noted, too, that both unfertilized and fertilized eggs lost potassium slowly

(1.5 to 8% in two hours) on standing in sea water, and that eggs in K^+-enriched (5 ×) artificial sea water accumulated K^+. In experiments with radioactive cations Brooks (1939) noted an increase in the accumulation of radiosodium upon fertilization in eggs of *Urechis caupo*. E. L. Chambers *et al.* (1948) reported that the rate of exchange of K^{42} increased 7 to 13 times upon fertilization in eggs of *Strongylocentrotus purpuratus* and *Arbacia punctulata*. They considered (see also E. L. Chambers, 1949) that only 20 per cent of the K is readily exchangeable in the unfertilized egg and 85 to 100 per cent in the fertilized egg. The rate of exchange of "freely diffusible" K is therefore concluded to be two to three times more rapid in the fertilized egg. While there are some evident differences in the results of the various experiments cited, the indications are that there are likely to be changes in permeability to certain ions, occurring upon fertilization, that may correlate with the changes in electrical potential.

On the other hand, the electrical changes might rather be due to alteration in the mechanical properties of the egg membrane. The large potential initially seen on penetrating the unfertilized egg was about equal in magnitude to the largest steady potential attained after fertilization. The decline from the initial value has been interpreted as being caused by imperfect sealing of the plasma membrane around the shaftlet of the microelectrode. It is therefore possible that the initial decrease upon fertilization indicates a further loosening of the seal and that the subsequent rise of membrane potential only reflects formation of a better seal. The various physical changes initiated in the fertilization reaction and discussed above might well be implicated in an alteration of the membrane seal around the electrode.

The electrical constants of the egg membrane

The measurements of membrane resistance and capacity were in the present experiments subsidiary to the use of the square pulse technique for ascertaining penetration of the egg surface by the microelectrodes. Therefore the results are chiefly indicative of the orders of magnitude of these values, subject to a more extensive study. However, the measurements can throw some light on the more detailed interpretation of the membrane potential and will be discussed largely in this context.

The unfertilized egg

Most cells thus far adequately studied have membrane resistances in the range of 1000 ohm-cm.^{2} However, the values range from as low as 0.1 ohm-cm.^{2} (rostral membrane of the electoplaque of the eel; Keynes and Martins-Ferreira, 1953) to as high as 20,000 ohm-cm.^{2} (activated *Fundulus* egg; Kao, 1955). The membrane resistance of *Asterias* eggs (average: 3,100 ohm-cm.^{2}) indicates that ionic transport is rather low across this membrane. In a tabulation of the membrane resistance of various cells, Cole and Curtis (1950) give an estimate that the membranes of both sea urchin and starfish eggs have infinite resistance. Earlier, however, Cole and Cole (1936a) pointed out that the method of measurement was not appropriate for determinations of membrane resistance. A 2% change in the estimate of relative cell volumes to that of external fluid would have led to a calculated value of membrane resistance as low as 25 ohm-cm.^{2} Rothschild (1938) stated that while his attempts to measure membrane resistance of *Echinus* eggs were unsatisfactory, the value was probably no higher and perhaps lower than 10^4 ohm-cm.^{2} Furshpan (1955) estimated that the resistance which the sea urchin egg interposed in the microelectrode circuit of his experiments was about 1 megohm. From the surface area of these eggs (diameter, 75 μ; area 1.8×10^{-4} cm.^{2}) he calculated the membrane resistance as 180 ohm-cm.^{2} However, since no membrane potential was obtained in those experiments, it is unlikely that the electrode had penetrated the egg membrane and this might account for the low estimate. Cole and Curtis (1938) calculated membrane resistances from 0.2 to 10 ohm-cm.^{2} for unfertilized and fertilized *Arbacia* eggs from measurements of single eggs in a small capillary but were forced to discard them because the assumption of no parallel leakage also gave unreasonable values for the membrane capacity. Allen, Lundberg and Runnström (1955) report a resistance of 8.7×10^5 ohms for an egg of *Psammechinus* in a narrow (57 μ) capillary but consider that this may be in error by virtue of a leakage pathway provided between the surface of the egg and the walls of the capillary. They state (p. 178) that in later experiments Lundberg (1955, unpub.) has obtained a value of 1350 ohm-cm.^{2}

Passage of currents of 0.5 to 1.5 μA through the *Asterias* egg, corresponding to current densities of 0.75 to 2.25 mA/cm.^{2}, caused marked decrease of the membrane resistance in 6 eggs. These results need further study, and if the phenomenon is established might yield valuable clues to membrane properties, since the change in resistance was, at least in some eggs, not accompanied by cytolysis. The relatively short pulses used in the experiments did not appear to cause any activation changes such as were reported in the experiments of Allen, Lundberg and Runnström (1955).

It is interesting to note that even with the highest currents employed, the relation between membrane current and voltage was strictly linear both for inward and outward currents. This is not the case in excitable tissues. Outwardly directed currents of relatively low magnitude initiate the changes of membrane potential inherent in the local response and spike and these are associated with a marked drop of membrane resistance, whereas inwardly directed currents tend to increase the resistance (Cole and Curtis, 1941). Thus, eggs of *Asterias*, although they undoubtedly undergo excitation by sperm in the form of the reactions of fertilization, evidently do not respond to electric stimulation in the manner characteristic of electrogenic excitable tissues.

It is of further interest that the maximum quantity of electricity in the square pulses used for the present experiments was about 5×10^{-5} coulomb/cm.^{2} The squid giant axon and other cells, however, are excited by 10^{-8} to 10^{-9} coulomb/cm.^{2} of membrane (Cole, 1949; Grundfest, 1952; Hodgkin, Huxley and Katz, 1952).

Effect of fertilization

If the initial decrease and subsequent increase of the membrane potential in the fertilized egg are consequences of altered ionic flux, the latter change should be reflected as a change of the membrane resistance. The most accurate measurements of this with respect to both magnitude and time course would be provided by study of the high frequency impedance. Such measurements were not done in the present experiments and the values derived from the square pulse technique apply, not to the initial period of fertilization, but to the stage when the fertilization membrane had been lifted and the membrane potential had reached its steady higher value. At this time the membrane resistance of the two eggs studied before and after fertilization (Fig. 8, Table 4A) was identical with the initial value. This may be taken to indicate that the fertilization membrane is a rather porous structure which offers relatively little impedance to ion movements. Cole (1928), Cole and Spencer (1938) and Cole and Guttman (1942) reported that upon fertilization of sea urchin or frog eggs there was no change in cortical resistance, and our data therefore also support this finding as regards at least a stage a few minutes after fertilization.

However, this constancy poses a dilemma which we have not been able to resolve. It was suggested earlier that the low value of steady membrane potential in the unfertilized egg, and its initial decrease and subsequent increase upon fertilization, might have been caused by alterations in the seal of the egg membrane against the wall of the microelectrode. This assumption would necessitate upward revision of the value of the membrane resistance of the unfertilized egg, perhaps up to double the calculated average of 3100 ohm-cm.^{2}, since the leaks decreasing the membrane potential to about half would be in parallel with the resistance of the membrane. However, the membrane resistance of the eggs seemed to be relatively independent of their steady membrane potentials (Table IVA), and therefore no attempt has been made in the present study to correct the calculated values of R_M. Nevertheless, the possibility remains that the membrane resistance of the unfertilized egg is higher than 3000 ohm-cm.^{2} and that the apparent absence of change after fertilization is fortuitous. The improved sealing of the membrane to the electrode might have increased the leakage resistance greatly and thereby have improved the accuracy of the measurement of R_M. In that case, the actual membrane resistance of the egg may have decreased upon fertilization. More extensive studies will be required to resolve this matter. However, the data, whether the resistance of the membrane is constant or decreases, differ from the finding that the membrane resistance of activated *Fundulus* eggs increases 4- to 7-fold (Kao, 1955). The difference may be explained by the fact that in *Asterias* the egg volume and surface remain nearly constant after fertilization, whereas the activated *Fundulus* egg shrinks markedly. The resultant diminution of surface may therefore lead to closer packing of the ion-permeable units of the plasma membrane (Kao, Chambers and Chambers, 1954), or the effective narrowing of pores in the membrane.

Cole (1938) reported a 100% rise in cortical capacitance of fertilized *Arbacia* and *Hipponöe* eggs and Cole and Spencer (1938) report 300% in *Arbacia*. In the two *Asterias* eggs studied before and after fertilization, the calculated membrane capacity after fertilization remained unaltered in one and decreased in the other. As noted earlier, the accuracy of the measurements of the time constant was not as high as is either desirable or attainable and this matter must be left open subject to future work.

SUMMARY

1. The paper describes electrical characteristics of the egg of the starfish *Asterias forbesii* as measured with a microelectrode penetrating the surface. The study included the effects of fertilizing the egg while the latter was impaled on the electrode.

2. It has been confirmed that penetration of the egg membrane cannot be indicated solely by the seeming visualization of the microelectrode at the center of the egg.

3. A method involving use of two microelectrodes is described for ascertaining penetration of the egg surface. One internal electrode delivers a current pulse and the other records the time course of the resultant membrane IR drop.

4. Contrary to the reports of many earlier investigations on echinoderm and other eggs a potential difference is found upon penetration of the unfertilized egg.

5. The potential difference at the time of penetration amounts to about 60 mv., inside negative, but this soon decreases to lower steady values ranging from − 10 to − 50 mv. in different eggs.

6. Upon insemination of the impaled egg the membrane potential abruptly decreases by 5 to 10 mv. when sperm are seen to have reached the egg, then rises during the ensuing 1 to 2½ minutes, as the fertilization membrane is raised, reaching a new steady value 5 to 20 mv. greater than that of the unfertilized egg.

7. The possible basis of these changes is discussed.

8. The internal K^+ of unfertilized *Asterias* eggs is from 17 to 21 times higher than that of sea water. The sodium determinations, while subject to larger error, indicate a concentration less than 5% that of sea water.

9. The membrane potential of either unfertilized or fertilized eggs decreases when the external K^+ is raised and returns to the original value when the excess K^+ is removed.

10. As in many other kinds of cells the potential is evidently a consequence primarily of the high internal concentration of, and permeability to, K^+, but the magnitude appears less than predicted by the Nernst equation.

11. The membrane resistance of the unfertilized egg averages 3100 ohm-cm.^{2}, but might be higher on the assumption of possible leaks around the microelectrode.

12. The measured resistance is unchanged after fertilization, but, on the assumption of the formation of a tighter electrical seal the actual membrane resistance would be lower.

13. The membrane capacity is of the order of 0.5 μF/cm.^{2}

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RELATIONS BETWEEN METABOLISM AND MORPHOGENESIS DURING REGENERATION IN TUBIFEX TUBIFEX. II.

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Analysis of the relations between metabolism and morphogenesis requires that each set of processes be separated into component parts. Metabolism may be fractionated by means of agents of which the effects on particular enzyme systems are reasonably well known and the relation of the activity of such systems to morphogenesis may then be tested. In the annelid, *Tubifex tubifex,* morphogenesis during posterior regeneration may be measured fractionally and "rate of localization," "rate of early differentiation," and "rate of later differentiation" expressed quantitatively (Collier, 1947). It was found that oxygen consumption and loss of weight by starving worms proceed at a markedly increased rate during certain stages of regeneration, and that rate of oxygen consumption was correlated with "rate of later differentiation"; a metabolic (energetic) cost of differentiation was hypothesized and it was thought possible that this might be characterized by activity of particular enzyme systems. The present report concerns the effects of continuous poisoning by cyanide and by iodoacetate and also the effects of high oxygen tension, low oxygen tension, and complete lack of oxygen upon morphogenesis during posterior regeneration in the oligochaete annelid, *Tubifex tubifex* Mull.

Materials and Methods

The worms were handled and examined as described earlier (Collier, 1947). For high oxygen tension, gas from a tank was bubbled continuously through the water in which the worms were kept. Presence of a low percentage of carbon dioxide (about 5%) was found to have no effect on experimental results. For low oxygen tension, nitrogen or hydrogen was bubbled through the water at two-day intervals, the bottles being tightly closed between treatments. For strictly anaerobic conditions, hydrogen from a tank was first freed of traces of oxygen by passing it over platinized asbestos heated to a dull red; then it was bubbled continuously through wash bottles and experimental bottles in series. That the continual disturbance did not affect regeneration was ascertained by using a control set-up through which air was bubbled.

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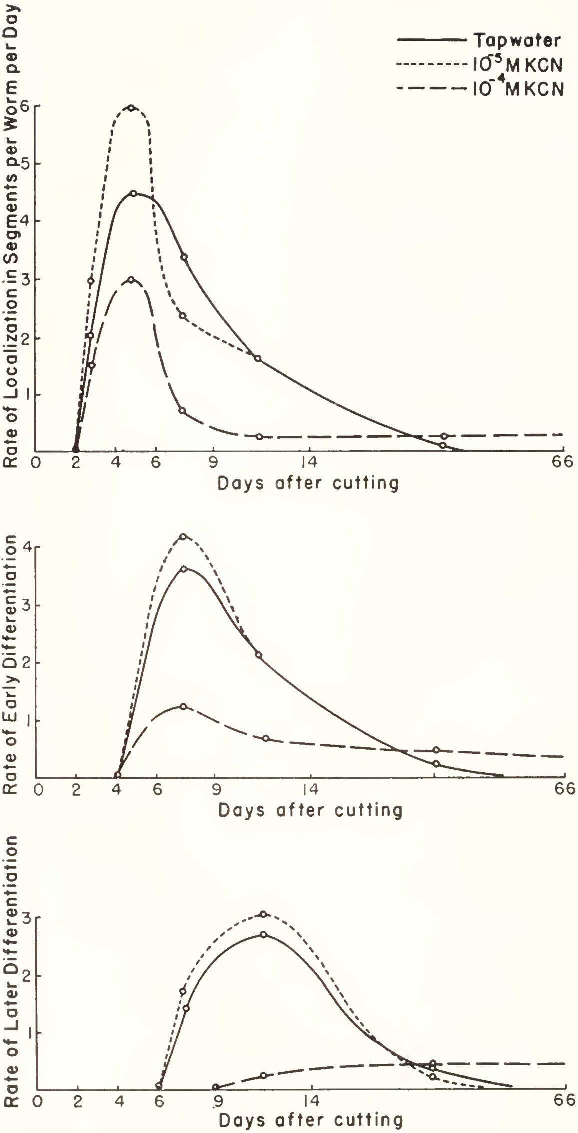

FIGURE 1. Effect of cyanide on rates of progress through various stages of regeneration. Mean deviation within each group of worms (thirty individuals) ranged from 0.3 to 0.5 segments per worm per day, increasing with time.

"Rate of localization" was calculated as increase in total number of segments per worm per day; "rate of early differentiation" as increase in number of segments showing some cellular differentiation visible *in vivo* under low power; and "rate of later differentiation" as increase in number of segments showing setae.

EFFECTS OF CYANIDE ON REGENERATION

Over 300 worms were used in experiments involving continuous poisoning by potassium cyanide. A group of thirty worms in 10^{-3} *M* KCN became inactive and showed heavy mortality after the second day. The last survivors formed blastemae, but no localization of new segments occurred in nine days. Worms in 10^{-4} *M* KCN and 10^{-5} *M* KCN survived well, one group in 10^{-4} *M* showing 60 per cent survival at 159 days. A few individuals showed abnormal regeneration: one double tail and seven with the new tail at an angle. Since three of these eight were in a control group, the abnormalities could not be attributed to effects of cyanide.

TABLE I

Effect of potassium cyanide on progress of regeneration: segments per worm per day

Days	Rate of localization					Rate of early differentiation					Rate of later differentiation				
	2–4	4–7	7–10	10–15	15–94	2–4	4–7	7–10	10–15	15–94	2–4	4–7	7–10	10–15	15–94
Worms in 10^{-5} *M* KCN	4.7	2.4	1.4		0.2	0	2.0	2.2		0.3	0	0	1.4		0.4
	4.8	2.4				0	2.0				0	0			
to tap water			↘ 1.1		0.2			↘ 2.3		0.3			↘ 1.4		0.4
Worms in 10^{-4} *M* KCN	2.0	1.9	0.5		0.3	0	0	1.1		0.4	0	0	0		0.5
	2.4	1.7				0	0				0	0			
to tap water			↘ 1.3		0.3			↘ 2.0		0.4			↘ 0		0.5
Worms in tap water	3.0	3.0	1.5	→0.8	0.2	0	2.0	2.2	→1.4	0.3	0	0	1.3	→1.9	0.4
to 10^{-5} *M* KCN				↘ 1.3	0.2				↘ 1.7	0.3				↘ 2.1	0.4

Data on rates of localization, early differentiation, and later differentiation are summarized in Figure 1. During the first six days the worms in 10^{-5} *M* KCN showed a significantly higher rate of localization than controls, while worms in 10^{-4} *M* KCN showed a lower rate. Later the advantage of the worms in 10^{-5} *M* KCN disappeared, but the disadvantage of those in 10^{-4} *M* KCN continued. At 66 days the worms in 10^{-5} *M* KCN and in tap-water had practically completed regeneration: all new segments were in later stages of differentiation and had grown to almost the size of the old segments. The total number of new segments was almost precisely the same for these two groups (average 41 per worm). At this time the worms in 10^{-4} *M* KCN still showed segments in the early stages of formation and the average total number of new segments was only 26 per worm. Growth of the new segments was poor. But at 159 days their condition was fully comparable with that of the others at 66 days. The total extent and perfection of regeneration were unaffected by treatment, but rate of regeneration was markedly affected.

The areas under the rate curves at the top of Figure 1 represent the average total number of segments localized per worm. For tap water and 10^{-5} *M* KCN, these areas between two days and nine days were 23 and 25, respectively, or not significantly different, despite the early rapid rate of localization in dilute cyanide. It may be presumed that the amount of cellular material available for localization of segments was a limiting factor, that this amount was unaffected by 10^{-5} M KCN and that the narrowness of the peak of rate was due to more rapid exhaustion of the material. The low rate of localization in worms in 10^{-4} M KCN and the fact that the area under the curve from two to nine days is less than half of that under the control curve suggests that availability of cellular material may have been decreased in 10^{-4} M KCN. The rapid decline to a very low rate of localization strengthens this suggestion.

Since rate of early differentiation during a particular interval of time should be limited by the number of segments localized, the differences in rates of differentiation were accounted for by the previous differences in production of localized segments. It appeared probable that the cyanide affected some process or processes occurring during or preceding localization, and had no direct effect upon later processes.

TABLE II

Regeneration in low oxygen

Days after cutting	Average number of new segments per worm		
	Nitrogen	Hydrogen	Control (air)
2 to 7	blastema	blastema	blastema
10	1.4	1.5	4.7
13	2.1	2.5	7.5 (setae)
16	3.7	3.0	9.8
19	5.0	5.1	11.7
23	6.0 (setae)		13.8
26	7.0		14.7

Mean deviation was about ± 1.0 segment up to 16 days, ± 2 thereafter.

An experiment was set up to test this idea. Five groups of thirty worms each were cut for regeneration and two groups were placed in 10^{-5} M KCN, two in 10^{-4} M KCN and one in tap water. At the end of seven days one group from each cyanide solution was transferred to tap water. Also, at the end of ten days the group in tap water was separated worm for worm into two comparable groups and one of these transferred to 10^{-5} M KCN. Data (summarized in Table I) in general confirm the supposition that cyanide affects localization and not later processes. Further, it is suggested that this effect is not upon mobilization of neoblasts, which, according to Krecker (1923) and Stone (1932), cease their metamorphosis and migration well before ten days. Rather the effect must be upon some process more directly concerned in localization.

EFFECTS OF LOW OXYGEN TENSION

If high oxygen tension acts as a stimulus to regeneration as suggested by Barth's work on Tubularia (1940), regeneration should be inhibited or retarded by low oxygen tension, while high oxygen tension should accelerate it. Exactly opposite

TABLE III

Observations on worms kept continuously under oxygen-free atmosphere

	18 hours	30 hours	42 hours
20 intact in tap water	dark red inactive	survival about 30%	survival 15%
20 intact in 10^{-5} M KCN	dark red inactive	survival about 80%	survival 45%
20 regenerating in 10^{-5} M KCN	dark red inactive	survival about 95%	survival 25%
20 intact in 10^{-6} M NaIAc	dark red inactive a few dead	no survivor	
Controls with air bubbled through tap water 20 intact 20 regenerating	normal	normal	survival 100%

results would be expected from the line of reasoning that in low oxygen tension there might be an increase in glutathione (*cf.* Barron, 1951) which, as found by Coldwater (1933), can increase rate of regeneration in Tubifex. One might then expect high oxygen tension to retard or inhibit regeneration.

One hundred and fifty worms were used in two experiments with atmospheres containing less than 4 per cent oxygen (Table II). No morphological abnormalities appeared, but there was a general retardation of regenerative processes.

Results of an experiment on survival of worms under more strictly anaerobic conditions (Table III) showed that this species cannot long endure complete absence of oxygen. Survival was significantly briefer in presence of iodoacetate.

TABLE IV

Effect of lack of oxygen on early stages of regeneration. (Each day access to air was permitted long enough to allow return of normal color and activity)

	2 days	4 days	5½ days	7 days
Oxygen-free				
Tap water	survival 7/30 1/7 with blastema	survival 0		
10^{-5} M KCN	surv. 30/30 24/30 with bl.	30/30 with small blast.	surv. 12/30 no blastema	survival 3/12
10^{-8} M NaIAc	no survivor			
Under air				
Tap water	30/30 with blastema			survival 30/30 aver. 19 new segments
10^{-5} M KCN	15/15 with blastema	aver. 11 new segments		survival 15/15
10^{-8} M NaIAc	15/15 with blastema			survival 15/15

Controls gave assurance that no materials from the apparatus or wash solutions had been responsible for destruction of the experimental worms.

For the purposes of studying regeneration an experiment was set up in which survival was improved by allowing a short period of access to air once a day (Table IV). The worms did produce blastemae at the usual time but regeneration proceeded no further and the blastemae disappeared. It appears that dilute cyanide improved survival but in the absence of oxygen did not show its accelerating action on localization.

The effect of anaerobiosis on later stages of regeneration was tested using worms which had regenerated for seven days under normal conditions (Table V). The worms kept under oxygen-free atmosphere (except for twenty minutes at 27 hours) showed practically no progress in regeneration, while in the control an average of 3.6 new segments per worm had been localized, 5.7 had undergone early differentiation, and 7.1 later differentiation. Regeneration here requires the presence of oxygen.

TABLE V

Effect of lack of oxygen on later stages of regeneration

	0 hours	47 hours
15 worms under oxygen-free atmosphere		survival 100%
Aver. no. segments in localization	7.0	6.2
Aver. no. segments in early differentiation	11.2	10.0
Aver. no. segments in later differentiation	0.6	1.7
Aver. no. segments total per worm	18.8	17.9
15 worms allowed to continue under air (Control)		survival 100%
Aver. no. segments in localization	7.0	4.8
Aver. no. segments in early differentiation	11.3	9.9
Aver. no. segments in later differentiation	0.6	7.7
Aver. no. segments total per worm	18.9	22.5

Mean deviation was about ± 1.0 segment.

EFFECTS OF HIGH OXYGEN TENSION

Concurrent with the experiments with low oxygen, two groups of thirty worms each were kept under an atmosphere of 95% oxygen and 5% carbon dioxide and a third group under 100% oxygen. Results were the same in all groups. The worms survived well for several days and formed blastemae, but regeneration proceeded no further and all worms had died by ten days. To test whether inhibition of localization by oxygen and stimulation of localization by 10^{-5} M KCN might be based on opposite effects on the same mechanism, experiments were set up in which worms were kept in 10^{-5} M KCN, 10^{-4} M KCN, and 10^{-3} M KCN under an atmosphere of pure oxygen (Table VI). The 10^{-5} M KCN partially counteracted the effects of oxygen both upon survival and upon regeneration; 10^{-4} M KCN was found to partially counteract the effect of high oxygen upon regeneration, but it did not even partially counteract the lethal effect. Accordingly, high oxygen tension had an effect on regeneration independently of its lethal action and, far from stimulating regeneration in Tubifex, very high oxygen inhibits it.

EFFECTS OF IODOACETATE

Since experiments in which worms were subjected to low oxygen tension and to complete lack of oxygen had suggested that glycolysis might be important for

TABLE VI

Simultaneous effects of high oxygen tension and cyanide on survival (in fractions) and regeneration (in average number of new segments per worm)

	2 days	4 days	6 days	9 days	14 days
Under oxygen Tap water	30/30 sm. blastema	23/30 0 seg.	4/30 0 seg.	no survivor	
10^{-5} M KCN	30/30 sm. blastema	30/30 2 seg.	28/30 3 seg.	21/30 4 seg.	no survivor
10^{-4} M KCN	30/30 sm. blastema	29/30 1 seg.	no survivor		
10^{-3} M KCN	no survivor				
Under air Tap water	30/30 blastema	28/30 4 seg.	28/30 13 seg.	27/30 23 seg.	27/30 31 seg.
10^{-5} M KCN	18/18 blastema	18/18 6 seg.	18/18 18 seg.	18/18 25 seg.	18/18 33 seg.
10^{-4} M KCN	15/15 blastema	12/15 3 seg.	10/15 9 seg.	10/15 11 seg.	10/15 12 seg.
10^{-3} M KCN	29/30 no blastema	no survivor			

survival and regeneration, worms were allowed to regenerate in various concentrations of sodium iodoacetate: 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M, and 10^{-8} M NaIAc. Five intact worms in 5×10^{-3} M NaIAc showed decreased activity after six hours, and were dead at thirty-six hours. In the other concentrations intact worms survived as well as but no better than the regenerating worms. The group of thirty worms in 10^{-3} M solution showed high mortality after three days, but the few survivors maintained normal morphogenesis. The worms in 10^{-4} M solution showed high mortality after six days, but six of the thirty worms survived for twenty-four days and maintained normal regeneration. Mortality in the other groups was low. Rates of progress through various stages of regeneration were very nearly the same for all concentrations of iodoacetate (Fig. 2), but whereas rate of later differentiation in tap water reached a peak between thirteen and seventeen days after cutting, the rate of later differentiation in each of the iodoacetate solutions

TABLE VII

Effect of dilute iodoacetate upon rate of oxygen consumption

Sample	Oxygen consumption in cubic millimeters per milligram of worms (wet weight) per hour	
Large worms (over 4.5 cm.)	0.12 in tap water	0.13 in 2×10^{-8} M NaIAc
Small worms (under 2.5 cm.)	0.14 in tap water	0.13 in 2×10^{-6} M NaIAc

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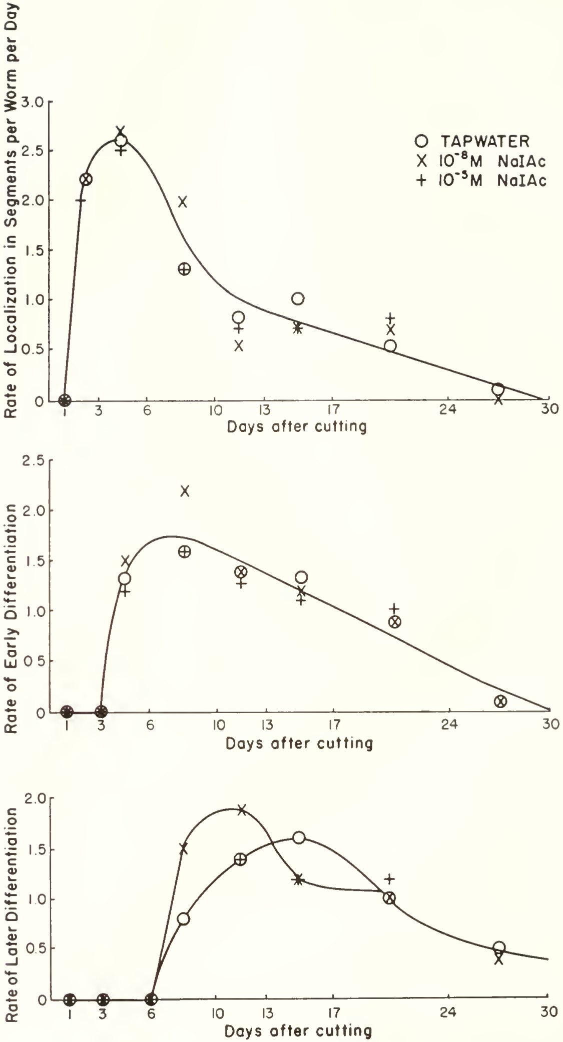

FIGURE 2. Effect of iodoacetate on rates of progress through various stages of regeneration.

reached its peak between ten and thirteen days after cutting. Iodoacetate appeared to accelerate later differentiation without affecting earlier processes. Because of the range of concentrations used, and because the extremely dilute solutions used here had maximal effect on differentiation, it may be presumed that iodoacetate poisons some process(es), inhibition of which allows further activity of some other process(es) in a system of multiple pathways of hydrogen and electron transfer (*cf.* Lipmann, 1954). The inhibited process might be glycolysis, while the reciprocally related process might or might not be concerned in the increased oxygen consumption previously found during the period of most rapid "rate of later differentiation."

A determination of the effect of sodium iodoacetate upon oxygen consumption of normal worms was made (Table VII). It is clear that iodoacetate had no significant effect on rate of oxygen consumption.

Discussion

It has been held that differences in rate of metabolism in the various parts of an animal may be the basis for production of morphological differences (Child, 1940; Hyman, 1940; Barth, 1938, 1940). Much of the supporting evidence comes from experiments on regeneration of hydroids, and the extremely rapid rate of regeneration here makes it difficult to distinguish between a factor influencing initiation of regeneration and one limiting later processes. In Tubifex, slow regeneration permits sufficient time for more detailed analysis. Since regeneration here is initiated even in the complete absence of oxygen, increased oxygen tension in the tissues at the cut surface is obviously not the primary stimulus nor is it even a necessary condition. Instead the availability of oxygen acts as a limiting factor in the progress of certain later processes in regeneration. However, the concept may be applied to morphogenesis during regeneration in Tubifex when used as Lindahl (1936) applied it in the echinoderm egg: differences in rate of particular fractions of metabolism may be the basis for certain initial processes in morphogenesis.

In the experiment in which worms with partially regenerated tails were subjected to lack of oxygen, the metabolism which supported vital processes did not support morphogenetic processes. Accordingly, regeneration must depend upon activity of some aerobic pathway. The fact that cyanide affected "rate of localization" indicates a cyanide-sensitive system important during localization. The fact that cyanide did not affect rate, extent or perfection of differentiation indicates that the particular system has little or no importance in relation to differentiation. Accordingly, on the basis of cyanide-sensitivity the processes supporting "localization" and "early differentiation" are distinct. Similarly, on the basis of sensitivity to iodoacetate the metabolic processes of "early differentiation" and of "later differentiation" are distinct.

During the period in regeneration before "later differentiation" appears, oxygen consumption was found to be only slightly, if at all, above normal (Collier, 1947). However, these worms lost weight almost twice as rapidly as controls, and this suggested an energetic cost of localization which was not reflected in oxygen consumption. The same applied to a possible cost of "early differentiation," but "later differentiation" was found associated with a markedly increased consumption of oxygen. Determination of respiratory sensitivity to cyanide showed that the increase was cyanide-stable. This contrasts with the findings of Bodine and Boell

(1934) for grasshoppers and of Sanborn and Williams (1950) for Cecropia moths, that the additional oxygen consumption during development is cyanide-sensitive although the respiration during diapause is entirely cyanide-stable. In fact it appears that the metabolic mechanisms of morphogenesis in metamorphosing insects (*cf.* Williams, 1951) can hardly be compared with those in regenerating Tubifex.

The presence of oxygen was found to be essential to "localization" but high oxygen tension inhibited it. There is no necessity for assuming that normal oxygen tension should establish optimal conditions for localization. Since these worms normally live partly submerged in mud, the optimum might be an oxygen tension lower than that established in very shallow mudless tap water under air. Fox and Taylor (1955) found this true for survival and growth of young worms in the laboratory.

Respiration as measured by the Warburg method was entirely stable to 10^{-4} *M* and 10^{-5} *M* KCN, but continuous exposure to these concentrations of cyanide affected "rate of localization." 10^{-4} *M* KCN was found to retard while 10^{-5} *M* accelerated "localization." Both concentrations counteracted the inhibitory effect of high oxygen tension.

It was considered that the accelerating effect of the more dilute cyanide solution is comparable to the often observed and seldom explained stimulation of various processes by other inhibitors in extreme dilution (*cf.* Commoner, 1940). Since in other cases the stimulation is effective upon the same processes which are inhibited by higher concentrations of the poisons, it was considered that the two concentrations of cyanide affected the same process in "localization." The concentrations of cyanide which activate proteinases *in vitro* are at least fifty times higher than 10^{-4} *M*, and were rapidly lethal to the worms (more minutely described by Hyman, 1916). Nevertheless cyanide here may have been effective upon the reactions of some metalloprotein other than those of the cytochrome system or of the haemoglobin in the blood of these worms. The antagonism of high oxygen damage by cyanide does suggest that the effects of high oxygen tension and of cyanide do meet somewhere, but if we assume that cyanide here is acting as an oxidative poison, then the cyanide-sensitive system cannot be responsible for any large proportion of the oxygen consumption: it may be off the main electron transfer pathway. The lethality of high oxygen tension also suggests an autoxidizable system that is off the main pathway (Gerschmann *et al.*, 1954). Whatever high oxygen affects, whether protein synthesis, concentration of particular normal or abnormal components, structural integrity, etc., it was at least partially counteracted in Tubifex by cyanide.

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SUMMARY

1. Continuous exposure of regenerating *Tubifex tubifex*, Mull. to cyanide has been found to affect "rate of localization" without affecting the ultimate extent or perfection of localization or of other morphogenetic processes.

2. Continuous exposure to iodoacetate has been found to increase "rate of later differentiation" without having other effects on regeneration.

3. Low oxygen tension was found to retard regenerative processes generally. In complete absence of oxygen, blastema formation took place but all subsequent processes were effectively blocked.

4. High oxygen tension blocked morphogenesis and also was lethal in from four to eight days. Both the inhibition and the lethal effects were partially relieved by concurrent treatment with cyanide.

5. It is concluded that the availability of oxygen limits the progress of later processes in morphogenesis without playing any necessary part in the initiation of regeneration in Tubifex.

6. It is indicated that metabolic processes supporting "localization," "early differentiation," and "later differentiation" are at least partially distinct from each other and from the metabolic processes essential to maintenance; that energy released in the promotion of particular morphogenetic processes must be released through particular enzyme systems; and that such specific release of energy is essential to the progress of morphogenesis.

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THE UPTAKE OF I^{131} BY THE THYROID GLAND OF TURTLES AFTER TREATMENT WITH THIOUREA[1]

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The chemical structure and relationships to goitrogenicity of several hundred compounds have been tested by a number of investigators, for example: the Mackenzies (1943); Astwood, Sullivan, Bissell and Tyslowitz (1943); Astwood (1943); Taurog, Chaikoff and Franklin (1945); Astwood, Bissell and Hughes (1945); McGinty and Bywater (1945) and VanderLaan and Bissell (1946). In general the active substances fall into one of three classes: 1) thiourea and its derivatives, 2) aniline derivatives, including the sulfonamides and other aminobenzene compounds, 3) thiocyanates and organic cyanides. The most active compounds tested possess a thiourea grouping or thioureylene radical —NH·CS·NH—. Replacement of the hydrogens of thiourea by methyl groups increases its activity, a fact which suggests the importance of the thio rather than the mercapto grouping for the activity of this class of substances. Extensive reviews concerning antithyroid agents have been published by Charipper and Gordon (1947), Astwood (1949) and Comsa (1953).

Vertebrates of every class have been tested for their reaction to antithyroid drugs. Such studies, however, for the most part, have dealt with mammals. Gordon, Goldsmith and Charipper (1943) made the first report of the use of inhibitors on the thyroid gland of cold-blooded animals. Since then a number of investigators have studied the effects of goitrogenic substances on poikilotherms. Lynn and Wachowski (1951) have published a comprehensive review of the literature dealing with the thyroid gland and its functions in cold-blooded vertebrates.

Little work has been done concerning the function of the thyroid in reptiles. Ratzersdorfer, Gordon and Charipper (1949), Adams and Craig (1951) and Fisher (1953) have reported the effects of antithyroid compounds on the lizards. Naccarati (1922) described the normal histology and gross anatomy of the thyroid gland of the turtle *Emys europa*, while Evans and Hegre (1940) studied seasonal changes and the effects of pituitary extract on the thyroid of *Chrysemys picta belli*. Greenberg (1948) was the first to investigate the effects of thiourea on the histology of the thyroid gland of the turtle. In her work immature specimens of *Pseudemys elegans* were used. Since then, Adams and Craig (1950) and Paynter (1953) have studied the thyroidal response to goitrogens in *Chrysemys picta picta*. Pastore

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(1950) investigated the effects of thyroid-stimulating and thyroid-inhibiting drugs upon the histology of the thyroid of *Clemmys insculpta* and *Graptemys geographica*. Dimond (1954) studied the reactions of developing snapping turtles, *Chelydra serpentina serpentina*, to thiourea.

Investigators who have worked on the thyroid gland of turtles are unanimous in pointing out, as an explanation for the observed irregular reactions to goitrogenic agents, what appears to be an inherent variability. This variability far exceeds that found in warm-blooded vertebrates. Uhlenhuth, Schenthal, Thompson, Mech and Algire (1945) working with the newt, *Triturus torosus,* claimed that such a high degree of variation does not seem explicable on the basis of the known physiological roles of the urodele thyroid and must be attributed to what might be called a general instability of the endocrines in cold-blooded vertebrates.

Some of the methods used to acquire a more thorough knowledge of the gland are: 1) gravimetric methods, based on changes in thyroid weight; 2) chemical methods, a quantitative as well as a qualitative study of iodine in the gland; 3) the use of radioisotope technique (radioactive iodine in the thyroid increases progressively with increasing dosage of TSH); 4) histological methods, which study mainly: a) epithelial height, b) staining reactions of colloid and cells, c) position and shape of nuclei and nuclear volume; 5) microhistometric methods. Uotila and Kannas (1952) have devised a linear measurement method, which permits quantitative determinations of the principal components of the thyroid tissue. The method appears to have the advantage of objectivity, simplicity and economy of time. This method was compared with the planimetric method and cell height measurement by Tala (1952) and found to give an accurate picture of the histological activity of the thyroid gland.

The use of radioactive iodine in experimental studies on the thyroid gland has added considerably to present-day understanding of the histophysiology of this organ. The early studies of Hertz, Roberts, Means and Evans (1940), Hertz and Roberts (1941), and Hamilton and Soley (1940), in which radioactive iodine was used for the first time in thyroid investigation, ushered in a technique which enabled more precise interpretation of the relationship between iodine metabolism, the thyroid and the hypophysis.

Tracer studies, using radioiodine after treatment with thyroid-inhibiting and thyroid-stimulating substances, are perhaps the most widely applied tools in thyroid investigation today. These recent developments are being used extensively in studying the thyroid function in mammals and are now being employed to some extent on cold-blooded vertebrates. However, as stated above, very few experiments have been performed on the reptiles. This is a particularly significant gap in our knowledge, in view of the fact that reptiles are considered as the stem of the birds and mammals in the vertebrate scale, and as the only cold-blooded amniote. One of the main reasons for this neglect has been the difficulty in performing thyroidectomy in these animals. The recent development of an effective "chemical thyroidectomy" opens new opportunities for research in this field. Already, some attempts have been made at correlating cell height, dry weight of the thyroid and the histological picture with the uptake of radioiodine in cold-blooded vertebrates such as salamanders (*Desmognathus fuscus* (Rafinesque); Fisher, 1953) and in the turtle *Chrysemys picta picta* by Paynter (1953). The results obtained in these experiments point again to the greatest difficulty encountered in the study of thyroid function at this level, namely, the astonishing variability. The present work is an attempt at investigating some of the factors which may influence this great variability in the function of the thyroid of the turtle *Chrysemys picta*. To this effect a study was made of the possible correlations between radioiodine uptake and colloid level, percentage epithelium, cell height and dry weight of the thyroid, in normal and in treated animals; an endeavor was also made to determine the thyroid/serum ratio, when organic binding is blocked and when binding is permitted.

Materials and Methods

Turtles with carapace length between five and seven inches were purchased from The Lemberger Company, Oshkosh, Wisconsin and the J. R. Schettle Frog Farm, Stillwater, Minnesota.

The experimental and control animals, totaling one hundred sixteen, were kept in large metal tanks which were arranged so that the animals had free access to running water or dry perches. The temperature of the room in which the tanks were located was kept as close to 75° F. as possible. All animals used in this series of experiments were denied food during the term of treatment since the amount of iodine in the food could not have been controlled.

The experimental animals were given subcutaneous injections of thiourea three times a week. The dosage, found most effective by Paynter (1953) for these experiments, was 0.25 cc./100 gr. of body weight of a 0.1% solution. After four, six and eight weeks of treatment the animals were injected with a tracer dose of three microcuries of carrier-free I^{131}, in 0.5 cc. of distilled water. The untreated control animals received the same dose of I^{131}. Three hours after administration of the radioactive iodine the animals were sacrificed. The plastron of each turtle was removed, blood was taken from one of the large vessels leaving the heart and allowed to clot. Immediately after exsanguination, the thyroid gland was removed and cut in half. The right half was cooled by placing it on dry ice. The left half, in 27 of the experimentals and 20 of the control animals, was immersed in isopentane, cooled by liquid nitrogen. Afterward, the glands, placed in individual tubes, on the surface of degassed paraffin, were transferred to the drying chamber of a freezing-drying apparatus (Altmann-Gersh technique; Gersh, 1932). Dehydration was carried out at − 40° C. and continued for a minimum of 72 hours. The system was then gradually brought to the melting point of paraffin by immersing the drying chamber in a beaker of water which was kept at constant temperature by placing it on a thermostatically controlled hot plate. The vacuum was broken only after infiltration was completed.

As for the remainder, 42 experimental and 27 control animals, the left half of the gland was placed on a glass slide and dried at 37° C. to determine dry weight. These glands were recovered by soaking in 0.025% solution of trisodium phosphate for 24 hours and in 10% formalin for three hours.

The right half of the gland, for each animal, was placed into glass homogenizers containing 6 ml. of ice-cold distilled water and one mg. of NaI as carrier, and homogenized immediately. The homogenate was deproteinized according to Somogyi (1945). To 0.80 cc. of homogenate, 0.10 cc. each of $Ba(OH)_2$ and $ZnSO_4$ were added, followed by vigorous shaking, and centrifuging. The supernate was removed with a pipette, three drops were placed on each of three glass sides. The same was done with the precipitate and a sample of the homogenate before deproteinization. These were dried at 37° C.

The serum was deproteinized by diluting a 0.05-ml. sample to 0.08 ml. with water containing 0.10% NaI and adding to it 0.10 ml. each of $Ba(OH)_2$ and $ZnSO_4$ as above. To determine the I^{131} content of the serum an aliquot of diluted serum (0.05 ml. diluted to 1.00 ml. with water containing 0.10% NaI) was dried on a glass slide.

Precipitable and non-precipitable I^{131}, as well as total I^{131} in both gland and serum, were measured with a Geiger-Mueller tube (window thickness 1.7 mg./cm.$^{-2}$) placed at a distance of 4 mm. from the object. The counts were also determined for the glands of both series, frozen-dried and oven-dried. Three determinations of one minute duration each were taken and averaged. The counts were brought up to the value on the day of injection by the use of decay factors which were based on the eight day half-life of I^{131}.

The glands were sectioned at 5 micra and those of the frozen-dried series were fixed by floating on 10% formalin and amphibian Ringer's solution and stained with Gomori's chrome alum hematoxylin and phloxin. Three sections were selected at 25%, 50% and 75% of each gland. Film strips of these sections were made and projected on white paper which served as a screen, using a magnification of × 100. Two lines, intersecting at obtuse angles in the form of an ×, were drawn in advance on the plane to which the image was to be projected. The image of the stained specimen was positioned so that its center fell approximately on the junction of the intersected lines. The outline of the follicles and colloid along the full length of the two lines was drawn. The segment of the lines covered by the entire figure was then measured in millimeters. Similarly, millimetric lengths of the segments covered by epithelium and colloid were determined. The total of the epithelium segments, divided by the whole length of the lines, gave the percentage of epithelium. The percentage of colloid was calculated in the same way. This linear measurement method of determining the principal components of the thyroid gland was devised by Uotila and Kannas (1952) and further tested by Tala (1952).

Observations

It is fairly well established from animal experimentation that antithyroid compounds of the thioureylene type owe their activity to their property of preventing the organic binding of iodine, or, what amounts to the same thing, the inhibition of the oxidation of iodide to iodine. Under the influence of these agents, iodide is still able to concentrate in the thyroid gland, but it remains in a reduced, ionic state. As a result of treatment with thiourea it should then be expected that radioiodine uptake would be higher in the experimental animals than in the corresponding control groups. This fact was well brought out in the course of the study; in all cases the counts were much higher in the treated animals than they were in the untreated. Analysis of the results based solely on length of treatment were much too variable to draw significant conclusions. Re-interpretation of these results on the basis of seasonal cycles gave a more valid picture.

A total of 72 animals were treated with thiourea; 52 of them received 18 subcutaneous injections over a period of six weeks and 20 received 12 injections over a period of four weeks. Forty-seven animals were used as controls and received three microcuries of radioiodine at the same time as the treated animals, three hours before they were killed. One set of controls was examined each time an experimental group was run.

An analysis of the results from two different points of view, 1) length of treatment and 2) seasonal factor, follows.

Length of treatment

From this point of view, the outstanding feature throughout the entire study is the great variability observed in control groups as well as in experimental animals. Taking the various correlations specifically the following can be reported.

Six-week series. The percentage epithelium was lower and the range of variation in percentage epithelium was considerably higher in experimental animals. The radioiodine uptake per unit epithelium and the range of variation in uptake were both higher in the treated than in the control group. The coefficient of correlation between the per cent epithelium and radioiodine uptake was − 0.38 for the experimental group and − 0.31 in the control animals. It becomes evident from the above figures that the correlations between per cent epithelium and uptake were practically nil in both treated and untreated groups.

The percentage colloid, radioiodine uptake per unit per cent colloid, and the range of variation were considerably higher in the treated animals than in the control group. The coefficient of correlation for the colloid and uptake was − 0.19 in the treated animals, while it was − 0.69 in the control group. In terms of colloid uptake the correlation was slightly improved in the treated animals but remained insignificant in the control animals.

Four-week series. There was no significant difference between the epithelium percentage and colloid level of experimental and control animals. The radioiodine uptake was much lower than in the six-week group and the range of variation was greatly reduced. The coefficients of correlation were as follows: per cent epithelium and uptake, control − 0.20, experimental, − 0.06; unit colloid and uptake, control − 0.10, and experimental − 0.27. The correlations in the four-week series were considerably improved but even if the range of variation was reduced it was still too high to permit significant correlations.

From the point of view of length of treatment it was impossible to establish significant correlations, due to the high range of variation. The correlations were poorest in the six-week series, but became somewhat improved in the four-week series. This was particularly true in regard to colloid level and iodine uptake.

Seasonal factor

During the course of this study the influence of a seasonal factor has been observed. Since the animals had been obtained from the supplier at three different times of the year, the three series therefore varied as to the time of the year during which the experimental work was done. When the results were analyzed in terms of seasonal cyclic activity, the correlation between epithelium or colloid and uptake was greatly improved and the variability was decidedly reduced, especially in the fall series. The first series was carried out during June and the beginning of July. The second series was carried out during November and the last series during the month of February and the beginning of March. As a result of such spacing, the

TABLE I

Radioiodine uptake per milligram dry weight. Difference in per cent of controls

Series	Mean count per milligram		% of control uptake
	Experimental	Control	
Winter	592.29	527.01	112.38%
Summer	985.34	534.85	184.22%
Fall	484.16	378.55	127.89%

possibility of a seasonal factor controlling the activity of the thyroid gland in the turtle was brought out, and this factor appeared to exercise its influence even in the treated animals.

Uptake per milligram dry weight. In the determination of radioiodine uptake, it was observed that 1) the counts per minute per milligram dry weight were higher in the treated animals than in the corresponding control animals, and 2) the counts for both experimental and control animals were highest during the summer and lowest in the fall series. This last point is a strong indication of the influence of a seasonal factor and is in agreement with Eggert (1935) who reported highest thyroid activity in June, in the case of hibernating lizards, and lowest activity during December and January. In this case the winter group, having been killed at the end of February and the beginning of March, would correspond to the resumption of the secretory activity. A summary of the results in per cent of control is given in Table I.

Thyroid/serum ratio. Measurements of the thyroid/serum ratios were made when organic binding was blocked with thiourea and when binding was permitted. Due to the high degree of individual variation, the time required for the maximum uptake, as determined by Paynter (1953), was found to vary considerably with

TABLE II

Per cent epithelium. Experiments in the order of their ranges of variation

Series	Seasons	No. of cases	Mean % epithelium	Maximum–minimum	Range	
					In points	In per cent of mean
Experimental	Winter 6 weeks	12	15.18	18.33–11.44	6.89	45.38%
	Summer 6 weeks	11	16.18	20.90–10.27	10.63	65.69%
	Fall 4 weeks	18	16.22	18.18–14.26	3.92	24.16%
Control	Winter	9	17.01	20.09–12.86	7.23	42.50%
	Fall	13	16.77	19.84–12.70	7.14	42.57%

each animal. Consequently a significant ratio could not be established. The effects of the seasonal cycle, however, could still be observed. In taking the counts per minute for the homogenate and comparing it with the counts for the serum the following results were obtained:

1) The summer group: in 92% of the experimental and 40% of the control animals, the thyroid homogenate had a higher count than the serum.

2) The fall group: in 43% of the experimental and 20% of the control animals, the thyroid homogenate had a higher count than the serum.

3) The winter group: in 36% of the experimental and 16% of the control animals, the thyroid homogenate had a higher count than the serum.

The results bring out very clearly the influence of a seasonal cyclic activity. The values obtained for the summer groups are almost three times as high as those for the winter groups and twice as high as the values obtained for the fall series. These differences appear to be directly correlated to the phase of activity of the thyroid gland in hibernating reptiles.

TABLE III

Colloid level. Difference between ranges of controls and experimentals

Season	Series	Colloid level Maximum minimum	Range	Difference between experimental and control ranges	
				In points	In % of control range
Winter	Control	75.50–63.12	12.38		
	Experimental	78.93–54.86	24.07	+11.69	+94%
Fall	Control	80.87–67.59	13.30		
	Experimental	78.74–60.67	18.07	+ 4.77	+35%

Per cent epithelium and iodine uptake. Two six-week series were carried out, one during late February and early March and the other during June and July, and one four-week series during November. The range of variation was too high to establish significant correlations between the per cent epithelium and radioiodine uptake, but the influence of the seasonal factor was nevertheless observed. The per cent epithelium in the treated animals was higher in the summer, with no significant difference in percentage for the fall group, but decidedly lower for the winter. In the control series, comparisons between percentage epithelium or colloid level are available only between the fall and winter groups. The summer control animals were not used for measurements. The range of variation in the three series gave a definite indication of a seasonal cyclic influence. Table II lists the per cent epithelium with the range of variation for the three seasons during which the work was carried out.

The uptake of radioiodine per unit per cent epithelium was considerably higher during the summer and lowest during the fall. Here again the results seem to indicate that the state of activity of the thyroid gland in the painted turtle corresponds

TABLE IV

Radioiodine uptake per unit colloid. Difference between ranges of controls and experimentals

Season	Series	Radioactivity Maximum minimum	Range	Difference between experimental and control ranges	
				In points	In % of control range
Winter	Control	122.74–10.88	111.86		
	Experimental	238.24– 6.58	231.86	+119.82	+107%
Fall	Control	197.51– 4.59	192.92		
	Experimental	129.76– 6.58	123.18	– 69.74	– 36%

rather closely to the seasonal cycle as described above and in agreement with the work of Eggert (1935).

The counts per unit epithelium in the treated animals were 383.63 for the summer, 335.79 for the winter and only 157.13 for the fall groups. Comparing the counts for the untreated animals the same general cyclic activity could be observed. The correlations, even though somewhat improved, still remained insignificant because of the range of variation.

Colloid level. The influence of the seasonal factor became more evident in the studies on correlation between colloid level and radioiodine uptake. The cyclic pattern was decidedly in accordance with the various phases of activity described for the hibernating reptiles. The mean uptake per unit colloid level was 79.91 for the summer, 65.31 for the winter, and 33.86 for the fall group in the treated animals.

The best evidence for this factor was brought out in the closer correlation be-

TABLE V

Colloid level experiments in the order of their ranges of variation

Series	Seasons	No. of cases	Mean % colloid	Maximum–minimum	Range	
					In points	In per cent of mean
Experimental	Winter 6 weeks	12	69.46	78.93–54.86	24.07	34.65%
	Summer 6 weeks	11	75.95	87.01–60.54	26.47	34.86%
	Fall 4 weeks	18	72.76	78.74–60.67	18.07	24.83%
Control	Winter	9	69.24	75.50–63.12	12.38	17.87%
	Fall	13	71.81	80.87–67.59	13.30	18.52%

tween the colloid level and the uptake of radioiodine in the fall group. The range of variation is at its lowest in both colloid level and range of uptake. Tables III and IV give the difference between the ranges of variation for colloid level and uptake in per cent of control range. It is to be observed that for the fall group the variation in colloid level for the experimental group was 35% above the control, as compared with 94% for the winter series. The range of variation of iodine uptake in experimental animals for the fall series was 36% below the controls and 107% above in the winter series.

From the above observations it was possible to conclude that in terms of length of treatment, the range of variation in uptake of radioiodine, either per unit of epithelium or colloid level, was much too high to establish significant correlations. When, however, the results were analyzed from the point of view of seasonal cyclic activity it became apparent that the gland was under the influence of a seasonal factor which exercised its control in treated as well as in untreated animals. More complete data on colloid level and uptake and variation are given in Tables V and VI.

Table VI

Radioactivity/unit % colloid. Experiments in order of time of treatment

Series	Seasons	No. of cases	Mean radioactivity per unit % colloid	Maximum–minimum	Range	
					In points	In per cent of mean
Experimental	Winter 6 weeks	12	65.31	238.24– 6.58	231.68	354.74%
	Summer 6 weeks	11	79.91	141.83–18.69	123.14	154.09%
	Fall 4 weeks	18	33.86	129.76– 6.58	123.18	363.79%
Control	Winter	9	57.99	122.74–10.88	111.86	192.89%
	Fall	13	40.61	197.51– 4.59	192.92	475.05%

Discussion

Antithyroid compounds may inhibit the normal function of the thyroid gland by acting directly on the thyroid itself at any one of the stages of hormone production: 1) the collection of iodide from the circulation; 2) the synthesis of thyroid hormone; 3) the release of hormone to the tissues.

Thiocyanate ions exert a unique effect upon the thyroid gland shared by no other substance yet known (Astwood, 1949). Animals treated with this substance have been shown to be unable to collect iodide from the circulation. Other substances, like thiourea and thiouracil, are believed to block an enzyme system, thereby preventing organic synthesis of the hormone, but having no effect on the "iodide trap."

Astwood and Bissell (1944) found that in rats under the continuous influence of thiouracil the iodine content of the thyroid rapidly falls to low levels and that the thyroid gland simultaneously enlarges. Astwood (1944-45) showed that animals thus depleted of iodine are still able to concentrate rapidly considerable quantities of iodine when injected with potassium iodide.

D'Angelo, Paschkis, Cantarow, Siegel and Riviero-Fontan (1951) have observed that despite uniformly decreased radioiodine uptake with chronic propylthiouracil treatment the total radioactivity in the thyroid eventually exceeds normal when sufficient hyperplasia has occurred to offset the limited uptake. The augmented radioiodine collections which result where the drug is withdrawn, however, are greater than would be expected from hyperplasia alone, and must result in part from increased avidity of thyroid tissue for the radioactive iodine. The augmented avidity for iodine upon withdrawal of the drug is demonstrable after periods of treatment too short to have caused hyperplasia, although it increases progressively with longer periods of treatment and consequently with thyroid hyperplasia before the goitrogen is withdrawn.

The results obtained in this study are in agreement with the above observations. The uptake of radioiodine was considerably higher in the animals in which binding was blocked as compared with those in which binding was permitted. This rapid uptake of iodine by the thyroid gland, reaching a maximum and followed by a discharge of the trapped iodine, varies with the type of animals used. In mammals, for example, the time required for maximum concentration may be as little as ten to fifteen minutes as reported by Hertz, Roberts, Means and Evans (1940) and Chaikoff and Taurog (1949).

In cold-blooded animals, however, great variations are observed in the reactions of the thyroid, and because of this great range of variability the maximum uptake in the turtle would seem to be controlled by a factor other than time alone.

In accordance with Adams and Craig (1950), Paynter (1953) and Dimond (1954) the results obtained in this investigation show that the reactions to thiourea in the turtles are not as profound as those reported in warm-blooded animals, and especially in mammals. The percentage epithelium was slightly lower in treated animals than in control animals, while the colloid was higher in the experimental than in the control animals. This could be explained by the fact that the experimental animals were injected with a tracer dose of I^{131} 24 hours after the last treatment with thiourea. This delay would allow the pituitary-thyroid axis to be restored to normal conditions, and consequently after a period of increased activity, the thyroid had stored a considerable amount of colloid.

Seasonal factor. A discussion of the seasonal physiology of any vertebrate immediately arouses an inquiry concerning the behavior of its endocrine glands, especially that of the thyroid and the pituitary because of the reactions to temperature by the former and the close association and control of the thyroid function by the latter.

In general, throughout the vertebrate classes, it may be said that if a species is inactive (hibernating) during the winter months, the thyroid is inactive at this time and will not become active until the animal resumes activity. Warm-blooded animals for example, which are active all winter, have thyroids reported to be more active during the cold season, in order to maintain their normal body temperature and BMR. The same animals have lower rate of thyroid activity during the summer, the temperature of the environment tending to prevent the loss of body temperature, thereby lowering the energy requirement for the maintenance of a normal BMR.

It can generally be said that the reverse is true in cold-blooded animals. However, the seasonal conditions of thyroids in cold-blooded vertebrate hibernators are less well known. Morgan and Fales (1942) reported that there are comparatively few observations on the full seasonal cycles of the thyroid of amphibians and some of these are conflicting. Several observers are in agreement concerning the seasonal condition of the thyroids in various species of frogs and toads, all of which are hibernators. In the main they report a winter phase of moderate activity and a summer phase of greatly lowered activity. Burger (1946), in his observations on seasonal conditions of the thyroid of the male of three species of urodeles, reports variations between animals and between individual follicles of the same gland. The three species, however, all showed a similar broad cyclic pattern in that activity was highest in the spring, lowest during the summer and moderate in the fall. Similar results have been reported by Miller and Robbins (1955) for the urodele amphibian *Taricha torosa* (*Triturus torosus*).

Although few in number, some studies of the seasonal cycle of the thyroid of reptiles have been made on hibernating and non-hibernating species. Eggert (1935) studied three forms of European Lacertas and reported hibernation beginning from the end of September reaching a peak in December and January when the gland is at its most reduced activity. Young animals resume their secretory activity in February and attain their highest activity in June. Seasonal variations in the thyroid of lizards have also been noted by Ratzersdorfer, Gordon and Charipper (1949) in *Anolis carolinensis*. In non-hibernating *Xantusis vigilis*, Miller (1955) has observed that the cycle is closely correlated with the various phases of the life history of the animal. He reports the lowest thyroid activity for the fall and an increase in activity during the winter. This increase in activity during the winter may be related to the fact that the animals are active and feeding during the coldest months of the year. The influence of the seasonal factor has also been observed in the cyclic activity of the thyroid in turtles. Evans and Hegre (1940), working with *Chrysemys,* obtained results which resembled those of typical hibernating forms even when the animals were fed at regular intervals and kept at room temperature (70° F.) throughout the fall and winter. This they claim would indicate that the thyroid gland of the turtle was under the control of a genetic factor. This factor exercises its influence independently of the temperature of the environment.

During the course of this study the influence of a seasonal factor has been observed which is in accordance with the results obtained by Uhlenhuth, Schenthal, Thompson and Zwilling (1945); Uhlenhuth, Schenthal, Thompson, Mech and Algire (1945); Evans and Hegre (1940) and Greenberg (1948). The experimental animals had been treated with a 0.1% solution of thiourea, a concentration claimed to be most effective by Paynter (1953). It would seem, however, that since turtles and reptiles in general appear to be more refractory to thiourea, a higher dose would produce more marked results. Despite the weak responses and the great range of individual variation, the results were more significant when interpreted in terms of seasonal activity.

The uptake of radioiodine by the thyroid gland was always considerably higher for the summer groups, treated and untreated, whether it was considered from the point of view of uptake per milligram dry weight; uptake per unit epithelium, unit colloid or thyroid/serum ratio. The uptake for the fall and winter series was much lower than that for the summer series. This is in agreement with Eggert (1935) who reports highest thyroid activity in June, in the case of hibernating lizards. The November series then would be nearing the most reduced thyroid activity which in lizards occurs during December and January. The winter group, killed at the end of February and the beginning of March, would then be expected to show resumption of secretory activity. The thyroid gland of the untreated control animals was observed to have a lower uptake of radioiodine per unit; however, the results were shown to follow the same general pattern as the treated animals. These results gave further evidence in favor of the seasonal cyclic activity.

The best correlations were found to occur in the fall series. The epithelium percentages of experimental and control animals were more closely related than in the other series; the range of variation in per cent epithelium was lower in the experimental animals; the correlation between epithelium and radioiodine uptake was definitely improved. The seasonal influence, however, was best demonstrated by the better degree of correlation between the colloid level and the radioiodine uptake. It was observed that the colloid level was lower in untreated controls than in treated thyroids. This may be due to the avidity of the treated thyroids for the iodine, since the injections of I^{131} were given 24 hours after the last treatment with thiourea. Considering the fact that the colloid is known to decrease during the active phase of the thyroid gland, it is not surprising that a lower colloid level was observed in the thyroids of controls for the February–March series than in the November series, which was during the phase of decreasing activity and colloid storage.

Since the turtles were kept in a room where a fairly constant temperature was maintained throughout the experiments, it would seem that the seasonal factor which regulates the cyclic activity of the thyroid in turtles is independent of the environmental temperature and probably is genetic in nature.

SUMMARY

1. The painted turtle, *Chrysemys picta* (Schneider), was treated with a 0.1 per cent solution of thiourea by means of subcutaneous injections, then injected with radioiodine in order to determine the correlation of percentage epithelium and colloid level with radioiodine uptake. The data obtained in this study were analyzed statistically in terms of length of treatment and the time of the year during which the work was carried out.

2. From the point of view of length of treatment, the correlations between the uptake of I^{131} and colloid level or per cent epithelium were very poor, due to the high degree of variability.

3. When these results were analyzed from the point of view of seasonal cyclic activity, the correlations were decidedly improved.

4. The best correlations were obtained in the fall group, where the percentage epithelium of experimental and control animals was more closely related; the range of variation was decidedly lower, and the correlation between colloid level and uptake of radioiodine much better than in the other series.

5. Further evidence in favor of the seasonal cyclic activity was found in the uptake per milligram dry weight, and in the thyroid/serum ratio.

6. This seasonal factor appears to be genetic in nature since it exerts its control independently of the environmental temperature and the effects of the drug.

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SOME FACTORS CONTROLLING THE INGESTION OF CARBOHYDRATES BY THE BLOWFLY[1]

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Diet selection and preference are commonly evaluated in terms of quantity of food consumed; however, measurements of intake alone give little information concerning the degree to which different factors participate in the regulation of ingestion. It is clear in the case of insects that a sequential contribution by various stimuli governs the finding of food, the initiation of biting or sampling, the continuance of feeding, and the termination of feeding. It is believed by some (*e.g.*, Dethier, 1953; Fraenkel, 1953) that stimuli which initiate sampling and which drive continued feeding are neither necessarily nor invariably correlated with nutritional values. Other workers, notably Kennedy (1953), believe that there is an important causal relationship between stimulating and nutritional characteristics. The present study is intended as a step toward the ultimate clarification of this problem.

Carbohydrates were chosen as test compounds because they do not stimulate the olfactory sense and because they represent all possible combinations of stimulating effectiveness and nutritional value. There are sugars which are stimulating but non-nutritional, stimulating and nutritional, non-stimulating but nutritional, and non-stimulating and non-nutritional. Sugars representing these categories were employed in the following experiments: (1) preference-aversion tests in which were recorded the volumes imbibed by flies given a choice between sugar and water or between one sugar and another; (2) individual feeding tests in which volume intake was measured in the absence of a choice situation; (3) tests of the sensitivity of the different chemoreceptor systems to stimulation; (4) measurements of the volume intake of mixed solutions; (5) longevity tests to ascertain the nutritional value of the various sugars at different concentration levels.

MATERIALS AND METHODS

Preference-aversion tests were conducted according to the procedure of Dethier and Rhoades (1954). In essence, the tests consisted of presenting groups of twenty flies, which had been enclosed in one-quart mason jars, with the choice of drinking from either or both of two J-shaped volumetric pipettes. The mean per capita fluid intake per twenty-four hours was calculated from the total volume of fluid taken from each pipette over a four-day period. In two-choice situations of this sort the intake of sugar can be compared with that of water or of any other sugar or sugar mixture.

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In order to ascertain the number of visits which were made to each pipette and the duration of each visit, the original apparatus was modified as follows. A silver wire was inserted into each pipette in such a way as to extend the entire distance from the large opening to a point just one millimeter short of the capillary orifice. Silver-conducting paint (DuPont Silver 4916) was then brushed in a thin line from a point near the large orifice to a point one millimeter short of the capillary orifice; here the painted line was extended around the circumference of the pipette so that a fly had to stand on the paint in order to drink. To the painted line near the large opening was soldered a silver wire. This wire and the wire from inside the pipette were each extended to the terminals of a Brush BL907 amplifier which in turn was connected to a BL202 recording instrument. Since the entire apparatus was intentionally unshielded, the two wires acted as antennae which picked up 60 cycle current from lights and various motors operating in the laboratory. Whenever a fly attempted to drink from a pipette, it closed the circuit between the conducting paint on which it was standing and the fluid and wire within the pipette. Since the 60 cycle current was then shorted out, the period of drinking appeared on the record as a straight line instead of alternating impulses (Fig. 1). The authenticity of records obtained by this method was confirmed by visual observation. At the same time the identity of the drinker was noted.

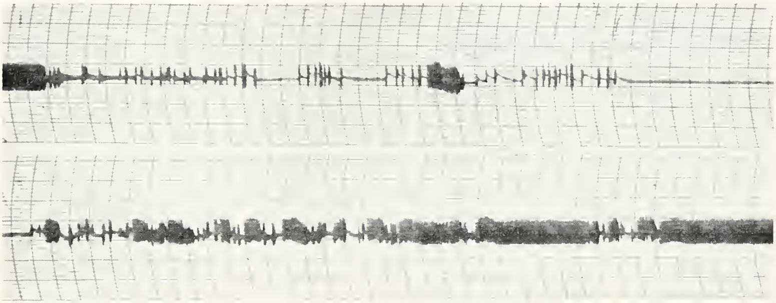

FIGURE 1. Typical example of automatically recorded periods of feeding. The thin line represents periods of feeding. Note that the fly has taken one long drink beginning at the upper right and continuing at the lower left. During the remainder of the time only brief samples were taken. Each curved line represents 5 seconds.

Finally, the fluid intake of individual flies was measured by direct analyses of sugar. For these measurements the flies were fed 24 ± 2 hours before testing on 0.1 M sucrose and then received neither food nor water until the experimental ingestion. At this time the flies were mounted on waxed sticks and individually fed on the test solutions. Some arbitrary criterion of repletion was necessary since a fly will continue alternately to extend and retract its proboscis almost indefinitely on some sugars, all the while taking small additional amounts. Repletion, therefore, was defined by the period of vigorous proboscis extension and active uptake. Usually a fly would feed continuously and actively for an initial prolonged period and then perhaps for an additional shorter period when its labellar hairs were brought into contact with the solution. This period of active feeding was usually rather sharply delineated, as indicated by the agreement of duplicate determinations on different groups of flies treated similarly. The standard deviation of replicate determinations of volume intake ranged between 0.377 μl for 1 *M* sucrose and 1.34 μl for 1 *M* fucose.

The determination of quantity ingested was accomplished by a sensitive spectrophotometric reaction for carbohydrates employing anthrone in concentrated sulfuric acid (Dimler *et al.*, 1952). For each determination the abdomens of 5–20 flies were ground, immediately after feeding, in 10 ml. of 5% trichloroacetic acid. The crop and intestine, which contain the ingested sugar, are located entirely in the abdomen after feeding. Equally large groups of flies similarly treated, but fed nothing, served as controls. After centrifugation, aliquots of the supernatant were diluted appropriately to produce concentrations from 30 to 200 μg. sugar per ml. One-ml. samples of these final dilutions were employed for the anthrone reaction and were compared spectrophotometrically with standards of the several sugars fed. The amount of sugar in the fed groups in excess of that in the unfed controls was directly convertible to volume since the concentrations of the solutions employed for feeding were known precisely. Longevity was measured with the same experimental set-up employed for preference-aversion testing.

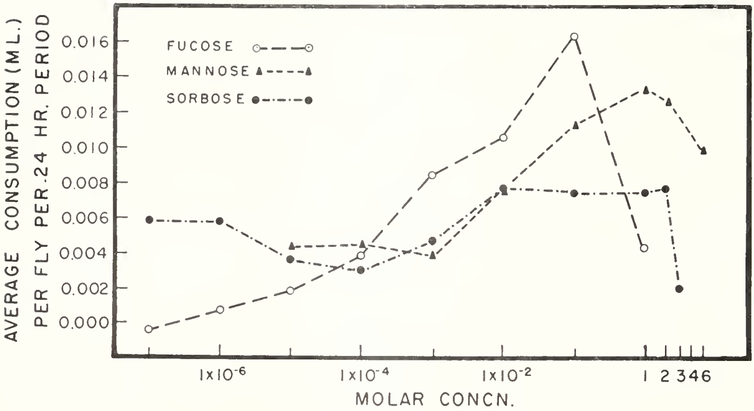

FIGURE 2. Preference-aversion curves for fucose, sorbose, and mannose.

PREFERENCE-AVERSION CURVES

The volume intake, as compared with water, was measured for each of the following sugars over the concentration range 1×10^{-7} *M* to saturation: fucose, sorbose, mannose. The results are summarized in Table I and Figure 2.

Fucose is a methyl pentose which is rather effectively stimulating for the tarsal chemoreceptors (median acceptance threshold = 0.087 *M*) but not utilized by the

TABLE I

Amount of solution consumed (ml./fly/24 hrs.) when sugar is paired with water

Molar concen.	Fucose	Water	Level (%) of significance of difference
1.0	0.0042	0.0035	—
0.1	0.0161	0.0012	0.1
0.01	0.0102	0.0031	0.1
0.001	0.0081	0.0041	0.1
0.0001	0.0037	0.0022	1.0
0.00001	0.0017	0.0033	—
0.000001	0.0008	0.0007	—
0.0000001	0.0000	0.0019*	0.1
	sorbose		
3.0	0.0020	0.0021	
2.0	0.0075	0.0036	0.1
1.0	0.0074	0.0027	1.0
0.1	0.0072	0.0036	0.1
0.01	0.0076	0.0050	0.1
0.001	0.0045	0.0030	1.0
0.0001	0.0030	0.0029	—
0.00001	0.0031	0.0012	—
0.000001	0.0058	0.0043	—
0.0000001	0.0059	0.0074*	5
	mannose		
6.0	0.0097	0.0024	5
4.0	0.0106	0.0016	5
2.0	0.0124	0.0011	1.0
1.0	0.0132	0.0023	0.1
0.1	0.0111	0.0022	0.1
0.01	0.0074	0.0032	0.1
0.001	0.0036	0.0046*	5
0.0001	0.0043	0.0054*	5
	rhamnose		
1.0	0.0068	0.0004	1.0
0.1	0.0067	0.0005	1.0
	lactose		
1.0	0.0212	0.0159	1.0
0.1	0.0091	0.0012	1.0
	D-arabinose		
0.1	0.0122	0.0004	0.1
	L-arabinose		
0.1	0.0035	0.0036	—

* These values represent the concentration range where water is taken in significantly greater amounts than sugar.

blowfly *Phormia regina* (Hassett, Dethier and Gans, 1950). Sorbose, a hexose, also stimulates the tarsal chemoreceptors (threshold = 0.14 M) although it is not utilized. Mannose, a hexose, is extremely poor in stimulating power (tarsal threshold = 7.59 M) but is nutritionally highly effective.

The curves describing the ingestion of the three sugars are substantially similar to those obtained by Dethier and Rhoades (1954) with the nutritionally adequate sugars glucose and sucrose. In each case there is a low concentration at which the sugar is not distinguished from water so that equal amounts of solution are taken from each pipette. Then, as the concentration is increased, a point is reached where more sugar than water is imbibed. This point represents a difference threshold. It occurs at a lower concentration than the tarsal acceptance threshold obtained by standard procedures. As the concentration is further increased there is an increase in the volume of solution imbibed until a maximum intake is reached, after which there is a marked decrease. A cursory examination of the curves reveals no relation between the volume intake and either the nutritional value or the relative stimulating effectiveness. Of the three sugars, the maximum intake is greatest for fucose and least for sorbose. None is consumed in as great quantities as glucose or sucrose.

Another characteristic of these curves is an inversion at very low concentrations where water may be taken in preference to sugar. With fucose, sorbose, and mannose the inversion occurs at 1×10^{-7} M, 1×10^{-7} M, and $1 \times 10^{-3} - 1 \times 10^{-4}$ M, respectively. Bimodal preference-aversion relationships of sugars were first noted by Beck (1956) in studies of the larvae of the European corn borer (*Pyrausta nubilalis* Hbn.). A re-examination of the raw data of Dethier and Rhoades (1954) reveals similar relationships. The meaning of rejection at low concentrations is not at all clear.

Individual Intake

When measurements were made of the volume of different concentrations of sugars imbibed by a single fly at one feeding (Table II) and the values plotted as a function of the concentration, the resulting curves differed in several important respects from the customary preference-aversion curves (Fig. 3). With the exception of fucose and sucrose there was no evident tendency for intake to decrease at high concentrations. There was, however, a marked tendency for intake to reach a plateau. On the other hand, regardless of the procedure employed for measuring intake, the weight of sugar consumed increased throughout the entire concentration range. There is no indication that the flies regulate the quantitative intake of sugar.

In comparing individual feeding curves with preference-aversion curves based upon four days of feeding the further difference is noted that the volume intake, while approximately the same in both experiments at high concentrations, at low concentrations is much smaller when measured individually than when measured in a two-choice situation. The fact that one experiment involves a two-choice situation while the other involves no choice has no bearing on the results because Dethier and Rhoades (1954) have shown that intake is the same in one-choice and two-choice situations. It seems possible to explain the difference on the basis of gustatory thresholds and behavior as affected by feeding. Earlier work (*cf.* Dethier and Chadwick, 1948) indicated that feeding elevates taste thresholds, and it seems reasonable to assume that the greater the ingestion of sugar the longer the taste threshold remains elevated (this assumption is borne out by experiments, soon to be published, on the determinants of taste threshold in *Phormia*). Furthermore, present data show that in general the volume ingested at a single feeding is a direct function of the stimulating effectiveness of the test solution. Hence, it might be expected that in preference-aversion experiments, after once feeding on 1.0 or 2.0 M sugar, the fly would not respond to the solution again for some time when it is encountered; and, furthermore, that when again ingested the solution will be taken in far lesser quantities as a result of the partially elevated threshold. Moreover, the number of encounters with the sugar solution is markedly reduced with flies feeding on 1.0 or 2.0 M sugar, since they are almost completely inactive for some time after ingestion of a large sugar meal. When 0.1 or 0.01 M sugar solutions are employed for preference-aversion tests, the post-ingestion duration of threshold elevation, the

TABLE II

Amounts of various sugars ingested at a single feeding

Sugar	Molar concentration	Number of animals	Mg./fly	Ml./fly $\times 10^3$	Duration* (sec.)	Rate ml./sec. $\times 10^5$	Approximate viscosity (centipoises)
Sucrose	2.0	20	8.96	13.0	90	14	—
	1.0	30	4.78	13.9	47	30	—
	0.5	10	1.80	10.5	43	24	—
	0.25	10	0.440	7.05	36	20	—
Glucose	2.0	15	4.92	13.7	61	26	—
	1.0	35	2.27	12.6	44	30	—
	0.5	15	0.820	9.11	38	25	—
Mannose	4.0	15	6.49	9.02	51	18	—
	2.0	15	2.97	8.25	40	21	—
	1.0	10	1.12	6.20	38	16	—
	0.5	10	0.268	2.98	25	12	—
Fucose	1.0	50	0.843	5.14	30	20	—
	0.5	15	0.580	7.08	32	35	—
Lactose	1.0	15	0.903	2.82	18	18	—
Sorbose	3.0	10	1.93	3.58	—	—	—
	2.0	20	1.09	3.04	—	—	—
	1.0	10	0.168	0.934	—	—	—
	0.5	6	0.0481	0.534	—	—	—
Sucrose	1.0	10	4.62	13.5	54	25	2.75
Sucrose 1.0M in:							
Glycerol	2.2	10	5.20	15.2	60	25	4.29
	5.4	10	2.86	8.34	50	17	7.79
	8.7	4	2.20	6.45	55	12	48.5

* Duration times were recorded for fewer flies than were employed in ingestion determinations.

period of quiescence, and the interval during which response fails upon contact with the solution are all shortened relative to the higher concentrations. The frequency of feeding is thereby increased. Thus may be explained the discrepancy of a higher daily intake of 0.1 *M* than 1.0 *M* sucrose, although at a single feeding much more is taken of the higher concentration.

The action of the above factors is again seen when the raw data of the preference-aversion curves of Dethier and Rhoades (1954) are analyzed on a day-to-day basis. It was found that curves based solely on the first 24-hour intake were displaced to the right, that is, the maximum intake occurred at very high concentrations. For subsequent 24-hour periods the intake of high concentrations drops while that of low concentrations gradually increases (see Dethier and Rhoades, Fig. 2).

The expectation of more frequent feeding on 0.1 *M* than 1.0 *M* sucrose was confirmed by automatic recordings of preference-aversion behavior. During the first eighteen hours of recording, 791 drinks were taken from 0.1 *M* sucrose and only 236 from 1.0 *M*. During the same period there were in addition 1,336 tentative drinks or taste samples of 0.1 *M* as compared with 898 of 1.0 *M*. The duration of drinking was approximately the same with each concentration; however, the volume imbibed per drink of 1.0 *M* was slightly more than twice that of 0.1 *M*. The rate of intake was, therefore, greater in the case of 1.0 *M*. It was also noteworthy that over the entire 18-hour period there was no marked decrease in the number of drinks of 0.1 *M* per hour, but the number of drinks of 1.0 *M* per hour had decreased by 80% at the end of 12 hours. The number had reached 0 at the end of 17 hours.

Sugars Paired with Each Other

In all of the foregoing choice experiments the test sugar was paired with water. In the following experiments sugars were paired with other sugars at many different concentrations. The results are summarized in Table III. From a perusal of these data it may be seen that the results are in general agreement with what might have been expected from an examination of Figure 2. For example, it might have been predicted from Figure 2 that more of 1.0 *M* mannose than of 1.0 *M* fucose would be ingested because the curve for fucose is displaced to the left relative to the mannose curve. The prediction was verified when the two solutions were actually paired (Table III). Similarly, the relative volumes imbibed in other two-choice tests are in general agreement with the basic preference-aversion curves. On the other hand, the *absolute* volumes are not the same in the two types of experiments. Such a discrepancy is to be expected, because volume intake is dependent not only on the concentration of the test solution but on the concentration and identity of all other compounds to which the insect is simultaneously exposed. The total situation is the determinant. For example, it had previously been found by Dethier and Rhoades that the less preferred of two sugars in a paired test was treated as though it were water regardless of how much of it might have been ingested when it was presented alone. In every case here, with the exceptions of 1 *M* mannose paired with 1 *M* sorbose and 0.5 *M* mannose paired with 0.5 *M* sorbose, the same is true. The less preferred member of the pair is ingested at approximately the same level as water (*cf.* Tables I and III). Consequently, the sum of the two volumes ingested in a paired test is generally less than the sum of volumes of each sugar which would have been ingested when paired with water, unless, of course, the less preferred is

Table III

Volumes (ml./fly/24 hrs.) ingested when different sugars are paired (preferred sugar underlined)

No.	Solutions paired			Significance at 1% level
1	1.0*M* mannose 0.0077	vs.	1.0*M* fucose 0.0007	+
2	1.0*M* mannose 0.0112	vs.	1.0*M* sorbose 0.0054	+
3	1.0*M* mannose 0.0121	vs.	0.1*M* fucose 0.0045	+
4	1.0*M* mannose 0.0154	vs.	0.1*M* sorbose 0.0014	+
5	0.5*M* mannose 0.0113	vs.	0.5*M* sorbose 0.008	+
6	0.1*M* mannose 0.0017	vs.	0.1*M* fucose 0.0138	+
7	0.1*M* mannose 0.0065	vs.	0.1*M* sorbose 0.0084	−
8	0.01*M* mannose 0.0074	vs.	0.0001*M* fucose 0.0046	+
9	0.001*M* mannose 0.0048	vs.	0.0001*M* fucose 0.0059	−
10	0.1*M* fucose 0.0129	vs.	0.1*M* sorbose 0.0026	+
11	1.0*M* fucose 0.0000	vs.	1.0*M* sorbose 0.0034	+
12	0.01*M* fucose 0.00294	vs.	0.01*M* sorbose 0.00140	+
13	0.1*M* D-arabinose 0.0143	vs.	0.1*M* L-arabinose 0.0043	+

being tested at a concentration at which it is not normally consumed more readily than water. In this last case the total consumption in the paired test would equal the sum of the two sugars tested individually.

In previous pairing of sucrose with glucose and sucrose with sucrose the volume intake of the preferred member was greater than in sugar-water pairs when the concentration in question fell at the peak of the preference-aversion curve, less if it fell on the ascending limb (*i.e.,* low concentrations) of the curve, and equal if on the descending limb. In the tests reported here the volume intake of the preferred sugar in a pair generally equalled its intake when paired with water when the concentration in question fell at the peak of the preference-aversion curve.

Both sets of data (Tables I and III) suggest very strongly that volume intake is under sensory control, that is, that the stimulating effectiveness of a solution determines how much of it will be imbibed. Several aspects of the two-choice data underline the importance of the sensory rather than the nutritional characteristic of the sugar in regulating volume intake. Line 6 of Table III indicates a preference for 0.1 *M* fucose (non-nutritional) over 0.1 *M* mannose (nutritional). This result clearly indicates the choice of a stimulating sugar over a poorly stimulating one. The choice of 0.1 *M* fucose over 0.1 *M* sorbose (line 10), both sugars being non-nutritional, reflects the superior stimulating effectiveness of fucose at this level of concentration. The relative intake of two sugars at concentrations represented on the ascending limbs of the preference-aversion curves appears to be sense-controlled, the more stimulating sugar always being preferred (lines 4, 5, 6, 8, 10, 12). This conclusion is in agreement with the findings of Dethier and Rhoades (1954) relative to the intake of glucose and sucrose.

When comparisons are made which involve concentrations on the descending limbs of the preference-aversion curves, stimulating effectiveness alone is apparently no longer the sole controlling factor; hence, comparisons at these levels are more complex (lines 1, 11). For example, the preference for 1.0 *M* mannose over 1.0 *M* fucose (line 1) does not result simply from the superior stimulating effectiveness of mannose, for indeed fucose is the more stimulating; instead, the preference undoubtedly reflects a negative factor causing the decline in fucose intake (*cf.* Fig. 3) as being responsible for the preference of mannose in the two-choice situation.

ROLE OF SENSORY SYSTEMS

The foregoing results clearly implicate the sensory systems. There are three chemosensory systems (exclusive of olfaction) definitely known to be involved in the feeding behavior of *Phormia;* namely, the tarsal chemoreceptors, the labellar hairs, and the interpseudotracheal papillae (Dethier, 1955). The first two mentioned have been studied to a greater extent than the papillae, and most of the remarks regarding stimulating effectiveness in the foregoing section have been based on information so derived. However, on the basis of these studies alone mannose should not be imbibed at all, and certainly its preference-aversion curve should not fall between that of fucose and sorbose.

The difficulty was resolved by the discovery that mannose, while poorly stimulating to tarsi and labellum, was an effective stimulus for the papillae. Its effectiveness at this site explains quite satisfactorily other difficulties encountered in the foregoing section. Mannose is obviously accepted at high concentrations in preference to sorbose, and in preference to water because of its stimulating effect on the papillae. Even though it does not stimulate the tarsal and labellar hairs, except at very high concentrations, it gains access to the papillae as a result of the fly's extending and probing with its proboscis in its normal exploratory behavior and in the course of ingesting to satisfy its need for water.

The discovery of the stimulating effectiveness of mannose on the papillae led to a series of tests in which other selected sugars were applied to the three chemosen-

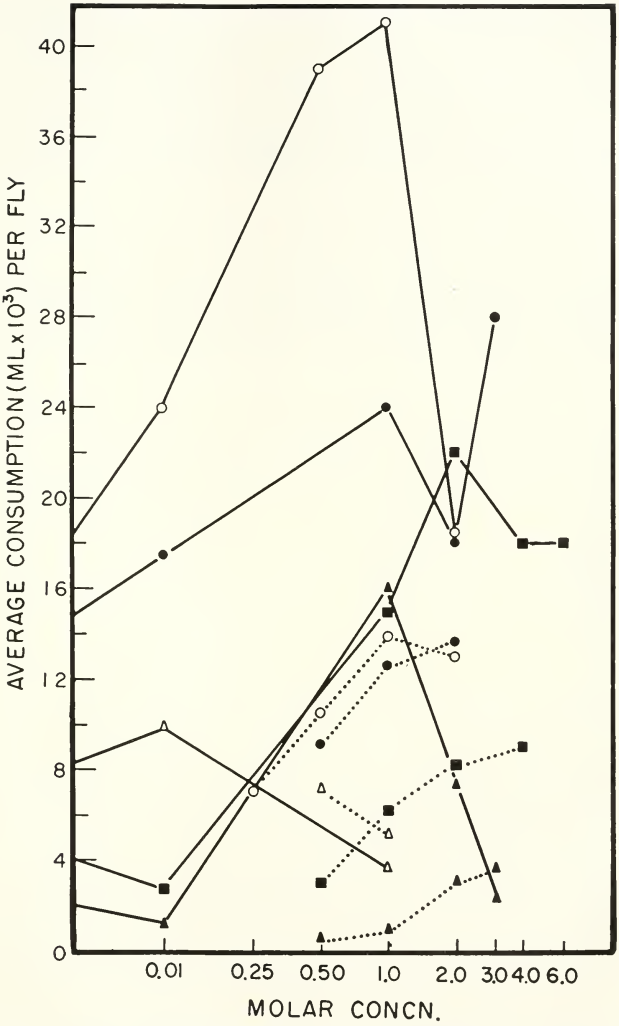

Figure 3. Comparison of ingestion measured by single feeding and by preference-aversion intake during the first twenty-four hours. Solid line, preference-aversion; dotted line, single feeding. ○ = sucrose, ● = glucose, ■ = mannose, △ = fucose, ▲ = sorbose.

sory systems. The results are given in Table IV. The most surprising result concerned L-arabinose, which was found to act as a repellent to the papillae even though it is acceptable in terms of its effect on tarsal and labellar hairs. This characteristic of L-arabinose was most unexpected. Clearly it stimulates the tarsal and labellar hairs, as a result of which the fly is moved to extend its proboscis and commence feeding. However, as soon as the solution comes into contact with the papillae, ingestion ceases abruptly. D-arabinose, by contrast, is acceptable to all three chemosensory systems and is consumed in appreciable quantities even though it is not utilized (Table I).

RELATION BETWEEN INTAKE AND NUTRITIONAL VALUE

From experiments in which different sugars were paired there were already indications that the stimulating rather than the nutritional characteristic of a sugar played a major role in regulating volume intake (*cf.* line 6 of Table III). The minor importance of nutritional factors, at least under experimental conditions, is revealed further by comparisons of the results of preference tests with sugar mixtures and the capacity of these mixtures for sustaining life. Two examples serve to illustrate the point, the behavior of flies with respect to glucose and D-arabinose and with respect to glucose and rhamnose.

TABLE IV

Effectiveness of selected sugars in stimulating the three chemoreceptive systems of Phormia

Sugar	Tarsal threshold (molar)	Labellar hairs	Interpseudotracheal papillae
fucose	0.087	+	+
sorbose	0.140	+	+
mannose	7.59	–	+
D-arabinose	0.144	+	+
L-arabinose	0.536	+	R
D-xylose	0.440	+	
L-xylose	0.337	+	–
rhamnose	–	–	–
ribose	–	–	–
lactose	–	–	–

(+ stimulating, – non-stimulating in all concentrations, R rejected)

Glucose alone at a concentration of 0.1 *M* supported life for 14 days (50% mortality); D-arabinose, for 3.5 days; a mixture containing 0.1 *M* glucose and 0.1 *M* D-arabinose, for 5.5 days. Survival on water alone averages three days (*cf.* also Hassett, Dethier and Gans, 1950). Yet in preference tests where glucose was paired with the non-nutritional mixture, flies consumed greater quantities of the mixture. Similarly, a mixture of 0.1 *M* glucose and 0.1 *M* rhamnose, which supported life for 8 days as compared to 3.5 days for rhamnose alone and 14 days for glucose alone, was consumed in greater quantity than glucose alone in a paired test (Table VII). Rhamnose paired with water was preferred slightly (Table I).

From these results it would appear that choices were made solely on the basis of the stimulating effect. There is no indication that either D-arabinose or rhamnose is repellent, since each is in fact preferred to water. While neither interferes with the stimulating effect of glucose on sense organs (Table V), both either are toxic or block glucose utilization.

Intake of Mixtures of Sugars

Sometimes the acceptability of compounds of very low stimulating power cannot be demonstrated in a two-choice test with water or by simple acceptance threshold determinations. Accordingly, the ruse has frequently been employed of mixing two sugars in order to detect suspected additive or repellent properties. Kunze (1927) and von Frisch (1935), for example, found that sugars which were acceptable to the honeybee were strictly additive. Unfortunately the technique is deceptively simple, and the results cannot always be relied upon to give the desired sensory information because of the occurrence of two phenomena which have not been given due consideration. These two are synergism and inhibition. They can be demonstrated most easily and convincingly by measuring tarsal acceptance thresholds to sugars and sugar mixtures. They also occur at labellar hairs. Tests for inhibition and synergism have not been made with interpseudotracheal papillae, but the occurrence of the phenomena at other sites indicates that an additive effect of sugars cannot be assumed as a matter of course.

Table V

Examples of inhibition revealed by ascertaining the effects of sugar mixtures on tarsal thresholds

Sugar	Effect	Sugar affected
mannose	inhibits	fructose
	does not affect	glucose, sucrose, fucose, maltose
sorbose	inhibits	glucose, fructose
fucose	does not affect	glucose, fructose
rhamnose	does not affect	fucose, glucose
	inhibits	fructose
D-arabinose	does not affect	glucose
mannitol	does not affect	fructose

For example, the median acceptance threshold for fructose is 0.0058; for glucose, 0.132; for an equimolar mixture of the two, 0.0078. In other words, the concentration at which the mixture is stimulating represents 0.0039 *M* glucose and 0.0039 *M* fructose. Even were the two sugars simply additive, they would not be expected to stimulate at this level. The fact that they do stimulate implies synergism. Mannose, on the contrary, when added to fructose inhibits it, that is, causes a ten-fold rise in the fructose threshold. This effect is not due to repellence because, for *Phormia,* mannose is preferred to water in all concentrations above threshold. Furthermore, mannose has no effect on such sugars as glucose, sucrose, or maltose.

The results of threshold tests with other mixtures are summarized in Table V. That the effects observed represent inhibition rather than repellence is further confirmed by the action of sorbose. Sorbose is stimulating in its own right, yet it causes an increase in the thresholds of glucose and fructose when mixed with them. Its action is revealed clearly in the following representative results (Table VI) where the per cent response of a sample of flies to various concentrations of glucose and of sorbose is compared to their response to a series of solutions which contain

0.5 moles of sorbose. In this case the stimulating effect of the mixture at low glucose concentrations stems entirely from the sorbose which is present. At higher concentrations of glucose, when the same amount of sorbose is present, there is little change in the stimulating effectiveness. Not only do the two sugars fail to add, but the stimulating effect to be expected of the high concentrations of glucose is absent. When, therefore, a smaller volume of a mixture of sugars is ingested than of either of the constituents alone, the result cannot always be ascribed to repellence, especially when both constituents can be shown in other tests to be preferred to water.

Galun (1955) has reported that all of the following sugars are repellent to *Musca domestica:* D-xylose, L-arabinose, ribose, rhamnose, and sorbose. This conclusion is based, however, on the fact that the addition of any of these to an acceptable sugar causes a lowering of intake. Unless the sugars can be shown to have a repellent effect when compared with water, the possibility of inhibition cannot be overlooked.

In the present studies some of the results of preference tests with sugar mixtures can be understood in terms of inhibition. For example, the volume intake of a mixture of mannose and glucose is greater than that of glucose alone, as would be expected (Table VII). In contrast, the intake of a mannose-fructose mixture, as compared with fructose alone, is not so great as would naturally be expected. Similarly with mixtures containing rhamnose there is a large increase in volume intake where the other sugar is glucose but no appreciable increase where the other sugar is fructose or sucrose (Table VII). This finding is in agreement with the threshold data (Table V) which indicate that rhamnose inhibits fructose but not glucose.

Table VI

Effect of sorbose on glucose threshold

Molar concen. of glucose solutions	0.0625	0.125	0.25	0.50	1.0
Per cent response	0	5	15	50	80
Molar concen. of glucose solutions containing 0.5 *M* sorbose	0.0625	0.125	0.25	0.5	1.0
Per cent response	45	50	60	50	65
Molar concen. of sorbose solutions	0.0625	0.125	0.25	0.50	1.0
Per cent response	5	20	45	60	80

The situation with regard to sorbose is not so clear although there is a tendency for the intake of sorbose mixtures to be less than expected on a purely additive basis. Such a result would agree with the postulated inhibitory effect of sorbose on glucose and fructose. It must nevertheless be emphasized that the effect of sugar mixtures on the papillae is not known, so that the results obtained in preference tests of mixtures cannot be fully interpreted in terms of demonstrated inhibition at tarsal and labellar sites alone.

The difference between repellence and inhibition, at least with *Phormia,* is a real one. Since the tarsal and labellar hairs of *Phormia* have been shown to consist of two receptors, one of which mediates rejection and one of which mediates acceptance (Dethier, 1955), a compound which is repellent might be expected to stimulate the rejection receptor while a compound which is an inhibitor might be expected to prevent stimulation of the acceptance receptor by interfering with the action of a stimulating compound on that receptor.

The only comparable study of mixtures on another insect is that of Wykes (1952), who measured ingestion of single sugars and mixtures of sugars by the honeybee. Although not explicitly stated, the experiment tested the hypothesis that the volume ingested of the four sugars examined, singly and in mixtures, was related to concentration by the formula $V = a + C$ where V is volume ingested at concentration C. For all four sugars, then, there was assumed to be a linear relationship between volume ingested and concentration, with a slope of unity and an intercept depending upon the sugar involved. Since, however, the units of volume employed were arbitrary and apparently were changed from one concentration to the next, this hypothesis was not tested directly; it was implicitly assumed in the analysis of ingestion of mixtures. The experiments on mixtures consisted of measuring the volume ingested of a solution containing equal proportions by weight of two to four sugars with a total sugar concentration of $x\%$ and testing the significance of the difference between this value and the average of the volumes ingested of each of the component sugars at $x\%$. For example, the volume ingested of a solution containing 8.5% sucrose and 8.5% glucose was compared with half the sum of the volumes ingested of 17% glucose and 17% sucrose. It was found, rather surprisingly, that the calculated and measured figures were not significantly different; hence, volume and concentration are linearly related, with a slope of unity for sucrose and glucose within the concentration range 17.1 to 51.3%. Similar experiments indicate that the same relationship is true for maltose. Furthermore, with one ex-

TABLE VII

Comparison of intake of mixed solutions with that of water or single sugars in a two-choice test

Concentration of each sugar in mixture	Vol. consumed ml./fly/24 hrs.	Water or sugar	Vol. consumed ml./fly/24 hrs.
0.05 *M* fucose and 0.05 *M* sorbose	0.0125	water	0.0019
0.5 *M* fucose and 0.5 *M* sorbose	0.0116	water	0.0030
0.05 *M* fucose and 0.05 *M* mannose	0.0213	water	0.0025
0.5 *M* mannose and 0.5 *M* sorbose	0.0184	water	0.0018
0.1 *M* fructose and 0.1 *M* mannose	0.0234	0.1 *M* fructose	0.0130
0.05 *M* glucose and 0.05 *M* mannose	0.0090	0.1 *M* glucose	0.0030
0.1 *M* glucose and 0.1 *M* mannose	0.0228	0.1 *M* glucose	0.0090
0.05 *M* glucose and 0.05 *M* sorbose	0.0099	0.1 *M* glucose	0.0162
0.05 *M* fructose and 0.05 *M* sorbose	0.0220	0.05 *M* fructose	0.0160
0.05 *M* glucose and 0.05 *M* rhamnose	0.0260	0.05 *M* glucose	0.0130
0.1 *M* glucose and 0.1 *M* rhamnose	0.0160	0.1 *M* glucose	0.0070
0.05 *M* fructose and 0.05 *M* rhamnose	0.0120	0.05 *M* fructose	0.0130
0.1 *M* fructose and 0.1 *M* rhamnose	0.0170	0.1 *M* fructose	0.0140
0.05 *M* sucrose and 0.05 *M* rhamnose	0.0180	0.05 *M* sucrose	0.0230
0.1 *M* glucose and 0.1 *M* D-arabinose	0.0210	0.1 *M* glucose	0.0080
0.05 *M* glucose and 0.05 *M* D-arabinose	0.0130	0.1 *M* glucose	0.0160

ception, these sugars in the mixtures tested are neatly additive in their effect on ingestion. The one exception was the glucose-sucrose-fructose mixture, of which more was ingested than was predicted (*i.e.,* there was synergism). This may reflect the synergism noted above on the tarsal threshold of *Phormia* for a mixture of glucose and fructose.

The data for *Phormia* relating volume and concentration, whether for intake at a single feeding or preference-aversion experiments, never present so simple a picture as Wykes's results. The only similarity may be the striking parallelism (with the exception of fucose) of volume increase from low to the optimum concentrations on a semi-log plot of ingestion at a single feeding (Fig. 3). Preference-aversion experiments on ingestion of mixtures probably are not comparable to ingestion as measured by Wykes; clearly, in the former case simple additivity of sugars in a mixture is not the rule.

THE FEEDING REACTION

Initiation of feeding. From the foregoing experimental facts and all other available information one can reconstruct, at least in part, the behavior pattern of the normal feeding reaction insofar as it is now known.

The normal pattern consists essentially of extension of the proboscis, spreading of the labellar lobes, sucking, and regurgitation. Apparently any one of three factors may initiate proboscis extension: (1) olfactory stimuli operating primarily through the antennae; (2) taste and possibly tactile stimuli operating through the tarsal receptors; (3) internal factors causing extension spontaneously. In the presence of vapors of an attractive nature a fly will extend its proboscis (*cf.* also Minnich, 1921). If the antennae are amputated, this faculty is impaired. Water (if a fly is thirsty) or specific carbohydrates can stimulate the tarsi with a resultant proboscis extension. In the absence of any specific external stimuli the fly will frequently repeatedly extend its proboscis in an exploratory manner.

The proboscis having been extended in response to any one or combination of these clues, the first parts which come into contact with the substrate are the long hairs of the aboral labellar surface. If the stimulus now received is favorable, the labellar lobes are opened, thus presenting the oral surface to the food. Sucking then commences. The labellar hairs, therefore, can regulate spreading of the lobes and sucking. They can also regulate extension, although under natural conditions it must be quite unusual for the hairs of the retracted proboscis to be stimulated. It could well be that in the event of the omission of an initial step in the normal sequence of stimulation, *e.g.,* stimulation of the labellar hairs before the proboscis is extended, the hairs trigger the missing step, in this case extension, before initiating the remaining steps. Control of the hairs over sucking is easily demonstrated. If, in a fastened fly, a drop of liquid just at the threshold of rejection is placed on the open labellum, it remains undisturbed, and the fly regurgitates into it. Surface tension prevents the fly from closing the labellum, and the feet cannot be employed to remove the drop because they are fastened. If now a single labellar hair is stimulated with a concentrated sugar solution (*e.g.,* 1 *M* sucrose), the drop, diluted with regurgitated fluids, is immediately swallowed.

Having opened the labellar lobes and commenced swallowing, the fly would no longer be in complete sensory control of the situation were it not for the interpseudotracheal papillae. Once the labellar lobes are opened the majority of the aboral hairs are no longer in contact with the solution. Even if they had been, the speed with which they adapt would certainly prevent a continual input from sugar stimulation from reaching the central nervous system. There is ample evidence that the papillae supply this defect.

Feeding can be monitored at four levels. If an odorous component of food attains a repellent level of concentration, feeding may be inhibited although ordinarily feeding will not have commenced under these conditions. Secondly, if the tarsal receptors are stimulated by unacceptable compounds, feeding is ordinarily stopped and the proboscis withdrawn. This reaction is, of course, the basis of all measurements of tarsal rejection thresholds. Thirdly, if the labellar hairs are affected by adverse stimuli, feeding stops. Fourthly, if the papillae are stimulated by unacceptable compounds, feeding is terminated.

As might be expected, these various levels of control are finely balanced. The coördination of sensory input from all of the receptor systems involved is extremely important for the proper accomplishment of feeding. Consider, for example, the relation between tarsal receptors and those on the mouthparts. Normally a fly will not commence feeding on a solution which has first been rejected by the tarsi. However, if arrangements are made to stimulate tarsi and mouthparts simultaneously with different solutions, the tightness of control of each system over feeding can be assessed. Application of sugar, however concentrated, on the tarsi will not cause feeding if a critical concentration of NaCl is placed on the labellum; but a low concentration of NaCl can be found which will be imbibed when the tarsi are stimulated with sugar, even though this salt is refused in the absence of tarsal stimulation. Conversely, concentrated NaCl on the tarsi will not prevent imbibition of sucrose applied to the labellum. The mouthparts, as might be expected, exert a tighter control.

On the mouthparts themselves the actions of the labellar hairs and interpseudotracheal papillae are usually coördinated. Experimentally either can be stimulated alone. The papillae alone are stimulated by inserting a micropipette between the closed labellar lobes or by rendering the hairs inoperative through waxing. The papillae are extremely sensitive to NaCl, and the application of salt by pipette causes an immediate cessation of feeding. However, it is sometimes possible to force salt imbibition by simultaneous stimulation of labellar hairs with concentrated sucrose. Swallowing is accomplished with great hesitation on the part of the fly if the salt solution is at all concentrated. Conversely, if the hairs are stimulated with NaCl while the papillae are stimulated with sucrose, feeding can be stopped, albeit somewhat slowly and temporarily. From the results of these two experiments it would appear that the papillae exercise tighter control over actual feeding than do the labellar hairs. The behavior of the fly toward L-arabinose confirms this. The hierarchy of command over sucking in ascending order is tarsi, labellar hairs, interpseudotracheal papillae. For proboscis extension and spreading of the labellar lobes, it is tarsi, labellar hairs. Stimulation of the papillae seldom causes proboscis extension or spreading of the lobes so that by means of a micropipette a fly can be induced to feed without extending its proboscis or expanding the labellum. In every case mentioned above the relative concentrations of the opposing stimuli are extremely critical insofar as the nature of the final response is concerned.

Control of volume intake. Although the various chemoreceptors generally work in harmony to regulate the economy of feeding response, the imbibition of liquids is only the beginning of a longer and more complex chain of events. Once the insect has begun to feed, it obviously does not continue indefinitely. Assuming that the substance being eaten or drunk is an acceptable one and that its stimulating effect (odor or taste) initiated feeding, what are the factors which ensure continuance of feeding and control of volume intake? It seems unlikely that the initial stimulation is alone sufficient to supply momentum for continued feeding without itself continuing, or, in other words, that feeding once started continues automatically until shut off. It is more probable that there is an additional factor which drives continuous feeding and another which terminates it.

Odorous foods not only can supply the initial stimulus but can also continue to stimulate for the duration of feeding. With odorless foods such as sugars, uninterrupted stimulation is also possible. If the fly is standing in sugar, the tarsal receptors can supply a continuous sensory input to the central nervous system until they become adapted. The principal stimulation from the mouthparts during feeding originates at the interpseudotracheal papillae because most of the labellar hairs are no longer in contact with the solution once the lobes have been spread. Even if the labellar hairs were in contact with the sugar, they adapt very rapidly. An experiment can be designed to show that, in the absence of any stimulation except that from the labellar hairs, complete adaptation of these hairs brings an end to feeding. For example, a fly which is not thirsty can be made to drink water if one or more of the labellar hairs are stimulated with sugar. Adaptation of the hair or hairs being stimulated causes feeding to cease, whereupon stimulation of different hairs which are still sensitive results in resumption of swallowing. From this result it would appear that a continual sensory input is indeed essential to uninterrupted feeding. Even stimulation of the tarsal receptors can drive feeding, and one way to force flies to imbibe non-stimulating fluids (*i.e.*, those which are neither acceptable nor repellent) is to apply sucrose to the legs. For many of the insects in which feeding reactions have been studied the prerequisite of sensory input is the rule (*cf.* Dethier, 1953).

Under natural circumstances a fly does not feed to full capacity upon first contact with an acceptable food but rather takes repeated samples. This behavior is graphically demonstrated by automatic recording (Fig. 1). In this way each new extension of the proboscis places the labellar hairs again in contact with the solution for fresh stimulation which imparts renewed impetus to feeding. At some point in the proceedings, however, feeding finally ceases; a definite quantity has been consumed. This volume is not constant but depends upon the hunger state of the fly, the nature of the food, and its concentration. Clearly neither gut capacity nor carbohydrate requirements immediately controls volume intake (*cf.* also Dethier and Rhoades, 1954). Thus, under normal conditions intake may cease long before the gut is fully extended. Furthermore, an isolated head does not drink equal amounts of all sugars. It takes in, for example, less sorbose than fucose and less fucose than sucrose, indicating control by structures of head alone.

An explanation which conforms most closely to the facts as now known is that intake is shut off by sensory adaptation. As an examination of Table II will reveal, the rate of imbibition and the duration of feeding increase with increasing concentration—up to a point. Since rate does increase with concentration and since maximum rates for different sugars are greatest for the more stimulating ones, there is reason to conclude that *rate* of intake is related to sensory input. It is highly probable, therefore, that the relationship prevails over the entire concentration range but that at a certain point (where measured rate declines) some negative factor intervenes. Since gram intake never declines nor becomes constant, the negative factor cannot be the amount of sugar or excessive or repellent stimulation. The cause must be sought in some other characteristic of the solutions. Increase in viscosity at high concentrations is one limiting factor. Measurements of rates of intake of a series of glycerol solutions of 1 M sucrose showed that rate decreases sharply with relatively small increases in viscosity. This finding is in agreement with the results which Betts (1929) had obtained in experiments with honeybees, where rate of intake declined sharply as concentrations of sugar exceeded 50% by weight. Betts concluded that viscosity was the limiting factor in this concentration range. At lower concentrations, however, she observed little change in rate with change in either viscosity or concentration. For the honeybee, temperature appears to exercise greater control over rate of intake than concentration does.

From the fact that duration of feeding increases with concentration one may infer that adaptation is one factor bringing an end to feeding. This inference is in accord with observed increases in adaptation time with increased concentration (Dethier, 1952). Additional evidence in support of this view derives from the observation that a fly which has ceased to feed on a given concentration may be induced to continue on a higher one and that a fly which has been feeding on a high concentration refuses to continue feeding on a lower one. In this respect isolated heads behave similarly. If the inference is correct, it would appear that flies adapt most quickly to fucose and less quickly to mannose, glucose, and sucrose, respectively, because this is the inverse order of duration of feeding.

Although the immediate cessation of imbibition can be explained in terms of adaptation, peripheral and central, and there is no evidence of action by internal factors at this point, it is almost certain that subsequent intake at various times after feeding to repletion is regulated by internal factors. These factors have been investigated and will be discussed in a latter communication.

SUMMARY

1. The ingestion of sucrose, glucose, fucose, sorbose, mannose, and lactose by the blowfly *Phormia regina* was studied by means of preference-aversion tests conducted for four-day periods; individual feeding tests; measurements of the sensitivity of the different chemoreceptor systems; measurements of volume intake of mixed solutions; and longevity tests.

2. The preference-aversion curves for all sugars studied indicated an increase in volume intake with increasing concentration up to an optimum point, after which there was a decrease in intake. At very low concentrations water was preferred to sugar.

3. Volume intake measured by individual feeding tests did not exhibit a pronounced decline at high concentrations. The difference between this finding and the one noted above resulted from the fact that flies ingested a maximum volume of concentrated solutions during the first visits to the pipette and then gradually ceased feeding altogether, while their ingestion of less concentrated solutions con-

tinued repeatedly over the entire test period. In all experiments the weight of sugar taken increased over the entire concentration range.

4. There is no relation between the amount of sugar taken and its nutritive value.

5. Volume intake is under sensory control. The coördinated actions of three principal chemosensory systems regulate the complete feeding reaction. The intake of mixed solutions depends upon the stimulating effectiveness of the mixture and whether or not any of the components exhibit synergism or inhibition. Some sugars show inhibition but no repellence.

6. The initiation of the feeding reaction is under sensory control. Continuance of feeding is dependent upon continuous sensory input. The rate of imbibition increases with concentration until viscosity begins to exert a restraining effect. The termination of feeding may be brought about by adaptation.

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THE CULTURE OF BRINE ALGAE [1]

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The biological productivity of the solar evaporation ponds used in salt manufacture is apparent even to the casual observer: they are a deep green or rich red. The ecological association in these ponds was the subject of several investigations (Peirce, 1914; Baas-Becking, 1928; Carpelan, 1953). Carpelan concluded that the productivity of the ponds, per unit area, was comparable to that of the ocean in its richer spots. But the ponds, being only half a meter deep, have a much more concentrated plankton community than does the productive zone of the ocean, which is 10 to 15 meters deep.

With the increased interest in recent years in mass culturing of algae for food production, the possibility of utilizing these evaporation ponds for such purposes has been considered. With this in mind, a laboratory study was undertaken on the cultivation of several of the unicellular algae which thrive in the ponds.

Pure cultures were isolated by the procedures described by Pringsheim (1946) with the added advantage of utilizing antibiotics, as suggested by Spencer (1952). The following five algae were studied:

Stichococcus sp.
Platymonas sp.
Dunaliella viridis
Dunaliella salina
Stephanoptera gracilis

These were isolated from the ponds of the Leslie Salt Company, on San Francisco Bay. The author is indebted to this company for many privileges during the course of the study.

SALINITY TOLERANCE

The determination of the range of salinity tolerated by the different algae under study is of primary importance. For this purpose several strains of the algae were inoculated into solutions of artificial sea water of varying concentrations.

Stichococcus strains numbers 1, 2 and 3, *Platymonas* strains 5 and 7, *Dunaliella viridis* strains 6, 8 and 9, *Dunaliella salina* strain 10, and *Stephanoptera gracilis* were studied. The strain numbers refer to the evaporation ponds from which they were isolated. Pond number 1 contained San Francisco Bay water (concentration less than sea water). The successive ponds contained increasing concentrations of brine to NaCl saturation in pond number 10.

Artificial sea water was prepared after Pringsheim (1946). Multiples of the sea water formula were used for preparing the desired concentrations. Concentrations up to ten-fold sea water were prepared; the solutions of concentration of X 5 and above were saturated with respect to the $CaSO_4$, excess solid being filtered off. Dilutions of sea water to $\frac{1}{2}$, $\frac{1}{10}$, as well as fresh water, were also used. All solutions were equally enriched with 50 mg. KNO_3, 10 mg. KH_2PO_4 and Hutner's trace elements mixture (Hutner *et al.*, 1950). Five-ml. portions of the various solutions were pipetted into test tubes and equally inoculated. The test tubes were kept in an inclined position under continuous illumination. Light was provided by several fluorescent lights suspended above the test tubes; its intensity was about 150 foot candles.

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Growth was estimated by cell counts after 16 days of culture. At this time the tubes in which no growth had taken place were re-inoculated with 0.1 ml. of the culture which was nearest to them in concentration. Thus, if a culture in the tubes of X 7 sea water did not grow, it was inoculated with the growing X 6 culture. The results of these experiments are given in Table I. The results are expressed as per cent of the growth in sea water, except for the values for *Stephanoptera gracilis*, which are expressed as per cent age of X 2 sea water medium. The results of the secondary inoculation are expressed as T (for trainable) and NT (for not trainable).

TABLE I

Relative growth of algal strains isolated from different salinities in media of different concentrations

Strain:	Medium Concentrations												
	0	1/10	1/2	1	2	3	4	5	6	7	8	9	10
Stichococcus sp.													
1.	30%	74%	170%	100%	NT								
2.	T	46%	135%	100%	15%	NT							
3.		T	90%	100%	40%	NT							
Platymonas sp.													
5.	77%	77%	90%	100%	90%	47%	NT						
7.		50%	100%	100%	125%	230%	60%	NT					
Dunaliella viridis													
6.	63%	82%	240%	100%	100%	95%	85%	50%	26%	NT			
8.	NT	34%	?	100%	225%	320%	230%	125%	100%	18%	T		
9.		T	60%	100%	210%	400%	320%	310%	120%	40%	T	T	T
Dunaliella salina													
10.		T	100%	100%	160%	220%	215%	185%	145%	80%	60%	55%	35%
Stephanoptera gracilis													
11.			T	12%	100%	120%	135%	67%	30%	T	T	T	

(Concentrations in multiples of sea water; T and NT refer to trainability or non-trainability of the algae for growth in the specific concentrations. Growth in sea water taken as base value for all strains except *Stephanoptera gracilis* in which × 2 sea water was taken as base value.)

From Table I it is apparent that the *Stichococcus* strains under study are primarily brackish water organisms. The *Platymonas* sp. appear to be more resistant to salt, although they too did not grow in the highly concentrated brines. The last three algae, all of the Polyblepharidaceae family, tolerate a wide range of salinity. Of these, *Stephanoptera gracilis* did not grow well at concentrations below X 2 sea water.

TEMPERATURE

To determine the temperature range for the organisms under study, the following procedure was adopted. Sea water was used for *Dunaliella viridis, Platy-*

monas sp. and *Stichococcus* sp.; *Dunaliella salina* and *Stephanoptera gracilis* were cultured in X 2 sea water. Media were enriched with nitrate, phosphate and Hutner's trace elements. Portions of sterile media were inoculated with the above mentioned cultures, five-ml. portions being pipetted into sterile test tubes.

Cultures were incubated in thermostatically controlled incubators, with light provided from two 15-watt fluorescent lamps. The test tubes were set on an inclined rack at a distance of 20 centimeters from the light source. Light intensity at the culture tube level was above 100 foot candles. After 10 days of continuous illumination, growth was estimated in the cultures by cell count. The results of the counts are given in Table II. The range of temperatures recorded by Carpelan (1953) for the evaporation ponds of the same region was from 8° to 20° in the winter, and 15° to 30° in the summer.

pH Effect

The effect of pH on the growth of the algae was investigated with the aid of media buffered with "Tris" (1, 3-propendiol-2-amino-2-hydroxymethyl). The pH range between 7 and 9 was investigated since this is the most common value for the sea water brines. Culture media were prepared as before except for the addition of 0.4 *M* "Tris" buffer; pH was adjusted to 7.2, 8.0 or 9.0. Five-ml. portions of the media were pipetted into test tubes and equally inoculated. The test tubes were kept inclined under continuous illumination. Cell counts were made after 9 days of growth. The results are recorded in Table III.

Table II

Effect of temperature on growth of brine algae

(Cells per cubic millimeter)

Temperature ° C.	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
8–10°	20	90	2,700	120	4,200
14–16°	200	560	36,000	340	24,000
24–26°	160	640		1,100	47,000
30°	450	1,800	35,000	1,000	40,000
35°	25	40	no growth	1,100	16,000

As seen from the table only *Stichococcus* sp. and *Platymonas* sp. show a definite preference for higher pH values. The other three algae do not seem to be very sensitive to pH changes in the range studied.

Phosphate Concentration

The optimum range of phosphate concentration for the algae under study was determined in an experiment in which 5 ml. of inoculated media in test tubes were enriched with graded quantities of phosphate. The media were buffered to pH 8 with .04 *M* "Tris" buffer. Nitrate and micronutrients were supplied as in the previous experiments. Cell counts were made after 10 days of cultivation under continuous illumination. The results are tabulated in Table IV. The optimum

TABLE III

Effect of pH on growth of brine algae

	Original pH	Final pH	Cells/mm.^{3}
Dunaliella salina	7.2	7.5	770
	8.0	8.1	1,300
	9.0	9.1	1,380
Stephanoptera gracilis	7.2	7.8	1,100
	8.0	8.1	740
	9.0	9.2	760
Dunaliella viridis	7.2	7.6	24,000
	8.0	8.0	15,000
	9.0	9.2	20,000
Stichococcus sp.	7.2	7.4	16,000
	8.0	7.9	25,000
	9.0	9.2	69,000
Platymonas sp.	7.2	7.5	570
	8.0	8.0	1,000
	9.0	9.2	2,270

range as seen in the table is in the same range of concentration which is recommended for use with several other marine algae (Koch, 1953; Ketchum and Redfield, 1938; Kylin, 1943).

NITROGEN SUPPLY

The growth of the algae on nitrate or ammonium as the nitrogen source was investigated. These are easily available forms of fixed nitrogen for mass cultivation of plants.

Media were prepared as before, buffered with "Tris" to pH 8.1. Phosphate and trace elements were supplied. Potassium nitrate was added in concentrations of 50 mg./100 ml. NH_4Cl was added in concentrations of 26.5 mg./100 ml. Cell counts were done after two weeks of culture under continuous illumination. In Table V the results of this experiment are summarized.

TABLE IV

Effect of phosphate concentration on growth of brine algae

(Cells per cubic millimeter)

KH_2PO_4 (microgram/ml.)	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
0	340	280	1,300	270	8,500
20	450	630	3,000	520	8,800
40	710	600	2,800	830	12,700
100	540	450	2,400	700	13,700
200	420	300	1,400	730	13,500
500	200	190	1,200	560	12,300

In another experiment, summarized in Table VI, using an unbuffered medium and higher concentrations of nitrate and ammonium (½ mg. N/ml.), the superiority of nitrate over ammonium for growth of all the investigated algae was clearly demonstrated. In this experiment urea was also studied; it was found to be a superior nitrogen source only for *Dunaliella salina.*

The ability of the algae to grow when supplied with organic nitrogenous substances as sole nitrogen source was also investigated. Media were prepared enriched with phosphate and micro-nutrients. The nitrogen source was added to a final concentration of 50 micrograms N/ml. The media were buffered with "Tris" to either 7.5 or 9.0. Cell counts were made after 12 days of culture, the results being tabulated in Table VII.

TABLE V

Nitrate and ammonium as nitrogen sources for brine algae in media buffered to pH 8.1

(Cells per cubic millimeter)

	Dunaliella salina	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
NO_3	1,700	1,500	4,700	6,000	48,000
NH_4	1,900	2,500	13,000	6,300	28,000

Contrary to expectation, uric acid, which is the main nitrogen form in bird excrement, does not serve as a good nitrogen source for *Stephanoptera gracilis* although this alga is usually found to bloom in high tide pools on rocks which are coated with bird excrement. *Platymonas,* which also blooms under similar conditions, can utilize uric acid. *Platymonas* alone was found to grow on all the organic nitrogenous substances studied; this might be a clue to an observation that this alga appeared to coat glassware in which animal or plant materials are kept under running sea water in the laboratory. *Stichococcus* sp. prefers nitrate to uric acid and asparagin; this is in contrast to the strains studied by Ryther (1954) which apparently grow better on uric acid and asparagin than on nitrate.

TABLE VI

Effect of nitrogen source on growth of brine algae

(Number of cells per cubic millimeter and final pH values indicated)

Nitrogen source	*Dunaliella salina*		*Stephanoptera gracilis*		*Dunaliella viridis*		*Platymonas* sp.		*Stichococcus* sp.	
	Cells	pH	Cells	pH	Cells	pH	Cells	pH	Cells	pH
NO_3	1,000	8.2	1,700	8.9	39,000	8.5	2,000	9.5	20,000	9.3
NH_4	600	6.4	700	5.0	6,000	5.5	400	6.5	10,000	6.9
Urea	1,400	7.8	500	7.5	6,000	7.5	300	8.3	8,000	7.8

The experiment was performed at two pH values with the hope of gaining information on the relative availability to the cells of the different ionic forms of the ex-

TABLE VII

Effect of various nitrogen sources on growth of brine algae

(Number of cells per cubic millimeter, and final pH values indicated)

Nitrogen source	Original pH	*Dunaliella salina*		*Stephanoptera gracilis*		*Dunaliella viridis*		*Platymonas* sp.		*Stichococcus* sp.	
		Cells	pH	Cells	pH	Cells	pH	Cells	pH	Cells	pH
Nitrate	7.5	870	7.8	1,370	7.9	25,000	7.8	530	7.8	28,000	7.7
	9	1,700	9.0	380	8.9	39,000	9.1	1,100	9.1	70,000	9.1
Uric acid	7.5	880	7.5	0		3,000	7.5	530	7.6	11,000	7.6
	9	1,100	8.7	0		4,500	8.7	1,300	8.9	17,000	8.8
dl-Aspartic acid	7.5	0		0		0		130	7.3	0	
	9	0		0		0		200	8.6	0	
Glutamic acid	7.5	970	7.9	530	7.8	0		500	7.7	0	
	9	530	8.9	270	8.8	0		500	8.9	0	
Asparagine	7.5	210	7.6	120	7.6	0		470	7.7	8,000	
	9	210	8.6	110	8.7	0		1,200	8.9	8,000	
Glycine	7.5	0		0		0		1,000	7.7	0	
	9	0		0		0		1,200	8.9	0	

amined substances. The growth on uric acid was found in general to be pH-dependent in much the same way as growth on nitrate. On the other hand growth on glutamic acid seems to show a reverse pH response in the case of *Dunaliella salina*.

ORGANIC NUTRIENTS

The ability of the brine algae to grow in the dark on several organic substances, as well as the effect of these substances on the growth in light, was investigated. Two organic energy sources were investigated, glucose and acetate. The effect of

TABLE VIII

Effect of organic substances on growth of brine algae

(Cells per mm.^{3})

Medium	*Dunaliella salina*	*Stephanoptera gracilis*	*Dunaliella viridis*	*Platymonas* sp.	*Stichococcus* sp.
1. Mineral only	700	970	6,250	630	27,000
2. Glucose	670	980	11,300	430	26,000
3. Acetate	580	870	6,200	770	—
4. YE* Glucose	700	750	9,200	420	16,000
5. YE Acetate	780	720	7,200	970	—
6. BH** Glucose	1,300	1,070	11,500	700	20,000
7. BH Acetate	670	900	9,500	920	—

* Yeast extract.

** Brain-heart infusion.

these was studied alone and in conjunction with complex mixtures such as yeast extract (YE) or brain-heart infusion (BH). The media were enriched with nitrate, phosphate and trace elements, and strongly buffered to pH 8.1 with 0.1 *M* "Tris" buffer. Glucose or acetate were used to final concentration of ½%, brain-heart infusion to .02% and yeast extract to .01%. Five-ml. portions of the inoculated media were pipetted into test tubes. Two equal sets of test tubes were maintained, one under continuous illumination and the other in the dark. The illuminated tubes were counted after 7 days of culture. The results are given in Table VIII. No growth was detectable in the tubes which were kept in the dark for three weeks.

The inhibition of growth of *Stichococcus* by the acetate is remarkable. The inhibitory effect of undissociated acetate on *Chlorella* is discussed by Myers (1951). In our case, however, the concentration of undissociated acetate is very low (about .0003 *M*). *Dunaliella viridis* seems to be stimulated by glucose, while *Platymonas* is stimulated by acetate.

SUMMARY

1. The conditions which the brine algae require for growth were found to be relatively simple. The high temperatures in the pond waters during the summer are well within the tolerance range of these organisms.

2. Simple nitrogenous substances and no organic supplements are required. No need for organic growth factors could be demonstrated under the conditions of cultivation used. Sea water, especially as it concentrates by evaporation, probably contains most of the required trace elements in sufficient quantity. It is possible that for mass cultivation of very dense algal suspensions, supplements of the micronutrients will be required.

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SOME ECOLOGICAL RELATIONSHIPS BETWEEN PHYTO- AND ZOOPLANKTON[1]

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The possibility of increasing human food resources by cultivation of unicellular algae is being rather widely investigated now (*cf.* Burlew, 1953). Two main approaches are considered: closed system cultivation of a pure algal culture under optimum growth conditions, and an open system, utilizing ponds. The closed system has the advantage of providing the maximal rate of photosynthesis and crop yield; however, it is expensive to install and maintain. The open system does not require elaborate installations and has the added advantage of utilizing ponds which may be constructed for a different purpose, algae being a by-product. Of such systems suitable for algal cultivation, sewage oxidation ponds are being studied. The growth of algae in these ponds is beneficial to the oxidation process, and the harvested algae save much of the nitrogen wastes and minerals which are otherwise poured into the sea (Gotaas *et al.*, 1954).

The solar evaporation ponds of the salt industry might also be utilized for this purpose. The natural productivity of these ponds per unit area approaches that of the open ocean (Carpelan, 1953), the difference being that the ponds are only one-half meter deep while the productive zone of the ocean is ten or more meters deep. This gives a very dense standing crop.

One of the economically limiting factors in mass cultivation of algae is their harvesting. In dense algal suspensions the volume of cells is still only a fraction of the total volume. To aid in the harvesting, suggested procedures have been sedimentation by slow settling (Smith, 1953) or flocculation of cells by added alum (Gotaas *et al.*, 1954).

Another possibility is utilizing grazing animals for the purpose of harvesting the algal cells. Raising fish in ponds is an old practice, the growth of plankton being accelerated by various fertilizers. However no attempt is usually made to maintain a maximal rate of production of the primary food, the algae.

In a preliminary investigation of the possibilities of the utilization of the sea water evaporation ponds of the salt industry, several unicellular algae which grow in this environment were studied (Gibor, 1956). We considered the possibilities of converting the algal crop to an animal crop by feeding a zooplankton grazer.

One of the important grazers in the evaporation ponds is the brine shrimp *Artemia*. This organism is easily maintained in the laboratory; its "eggs" (cysts) are readily available and can be kept in the laboratory for many years without losing their ability to hatch. We attempted to study the nutritive value to *Artemia* of several of the unicellular green algae which were isolated from the brines of the evaporation ponds. The algae used were:

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Dunaliella viridis
Dunaliella salina
Stephanoptera gracilis
Platymonas sp.
Stichococcus sp.

Dunaliella salina and *Stephanoptera gracilis* were cultured in sea water evaporated to half its original volume; the other three species were cultivated in sea water.

The feeding experiments were carried out as follows. *Artemia* cysts had been collected two years earlier from salt ponds of the Leslie Salt Company, and kept in a closed jar in the laboratory. Such cysts were suspended in water and centrifuged (in a clinical centrifuge) for several minutes. The light cysts were decanted off as suggested by Dempster (1953). The heavy cysts were re-suspended in merthiolate solution (1 : 1000, in water) for 10 minutes, then centrifuged. The merthiolate solution was decanted off and the cysts washed four times with sterile sea water to get rid of the merthiolate. Finally the sterile cysts were transferred into a one-liter Erlenmeyer flask containing sterile sea water and allowed to hatch.

The sterility of the cysts and larvae was determined by suspending a fraction of the sterilized cysts in sea water enriched with 2% yeast extract-glucose solution and incubating for several weeks. To further verify their sterility a dense cyst suspension (⅓ ml. eggs in one ml. enriched sea water) was also incubated. The autolysing cysts should supply additional growth requirements for contaminating bacteria. Both these tests were negative and indicated the adequacy of the sterilization method. The larvae were transferred when needed with a sterile Pasteur pipette into a test tube for addition to the algal culture. Ten to 20 larvae were introduced into one liter of a dense algal culture in a two-liter Fernbach flask. The culture was kept under continuous illumination, with continuously bubbling air. After 6 days the cultures were still green, indicating that until this time the quantity of food was not limiting the growth of the animals. At this stage the *Artemia* were harvested by passing the whole solution through a fine plankton net. The animals were fixed in 0.5% formalin in sea water. The control larvae in sterile sea water were dead of starvation at this time.

TABLE I

Size of Artemia salina fed on different brine algae for six days (average length exclusive of caudal furca)

Alga	Sterile culture	Non-sterile culture
Stephanoptera gracilis	2.3 mm.	2.8 mm.
Dunaliella viridis	2.1 mm.	2.8 mm.
D. salina	1.5 mm.	2.3 mm.
Platymonas sp.	1.5 mm.	2.0 mm.
Stichococcus sp.	0.7 mm.	0.4 mm.
None (unfed)	0.2 mm.	0.2 mm.

The results of the feeding experiment are shown in Table I.

Dunaliella viridis and *Stephanoptera gracilis* appear to be superior as foods to *Dunaliella salina* and *Platymonas* sp., while *Stichococcus* sp., is evidently a poor nutrient for *Artemia*. Consistent results were obtained in a second experiment except that growth on *Dunaliella salina* in this case was as good as on the other two Polyblepharidaceae.

For ecological purposes it seemed advisable to find whether these results also hold under non-sterile conditions. The apparent superiority of the Polyblepharidaceae might, for example, be due to the absence of a rigid cellulose wall; bacteria (in the *Artemia* gut) might aid in the digestion of the cellulose wall of *Platymonas* and *Stichococcus*. An experiment therefore was conducted under conditions identical to the first except for the use of unsterilized *Artemia* larvae. These results (Table I) show an improved growth in all cultures except *Stichococcus* sp. with the Polyblepharidaceae still showing better growth. Bond (1933) found that *Platymonas* sp. was slightly superior to *Dunaliella viridis* as food for *Artemia*. His criterion was the time in which *Artemia* reached the mating stage: on *Platymonas* sp. the required time was 28 to 29 days, on *Dunaliella viridis* 31 days. However in one of our experiments non-sterile *Artemia*, growing on *Dunaliella viridis*, were found copulating after 13 days.

To investigate whether *Stichococcus* is producing an inhibitor to the growth of *Artemia*, a mixed culture of *Stichococcus* sp. and *Dunaliella viridis* was inoculated with *Artemia* larvae. Good development of the larvae showed that no inhibitor was produced.

Microscopic observation established that the *Artemia* larvae do ingest *Stichococcus;* the deficient growth on this alga is thus not due to the inability of the animal to filter and ingest the smaller cells of this genus.

On the basis of the estimation of the *Stichococcus* crop and the population of *Artemia* in the evaporation ponds Carpelan (1953) concluded that *Artemia* utilizes only a small fraction of the crop of *Stichococcus*. Our results based on laboratory tests corroborate this opinion.

The observations made in the experiment on the nutritive value of algae for *Artemia* aid in understanding the ecological relationships in the series of evaporation ponds.

One of the striking facts observed in the salt ponds is the predominance of *Stichococcus* in brines of relatively low salinity (to about three-fold sea water). Both *Platymonas* sp. and *Dunaliella viridis* can be isolated from such low salinity brines, and both algae grow well in these concentrations. However, they are always overgrown by *Stichococcus*.

The finding that *Artemia* does not grow on *Stichococcus* suggested that the animal might act as a differential filter, ingesting the algae on which it grows well and leaving those on which it can not grow. The possibility of a differential mechanical effect was eliminated by observations on starved *Artemia* put into a *Stichococcus* suspension. As mentioned above, the animals fill their gut with this alga in a few minutes.

To determine whether live cells survive in the fecal pellets, sterile *Artemia* were fed on a pure culture of *Stichococcus*. After feeding for several hours the animals were washed by transferring them into a corner of a Petri dish containing sterile sea water. Use was made of the positive phototropic response of the *Artemia*. The fast swimming animals were collected from the opposite, light side of the dish and transferred to a second dish for repeated washing. After 4–5 such washings the animals were transferred into a depression slide containing sterile sea water and left for several hours. Fecal pellets accumulated in the depression slide. Single pellets were picked with a sterile Pasteur pipette and transferred through a series of sterile sea water droplets. The washed pellets were finally inoculated into test tubes containing several milliliters of sea water enriched with minerals, and kept under continuous illumination.

Test tubes in which growth of algae occurred were examined microscopically to determine whether we were dealing with the same algae as fed to the animal. Ten test tubes so treated were found to contain growing *Stichococcus* cultures. Clearly some cells survived ingestion. An ecological advantage for one algal species over another might be established by even a slight difference in such ease of digestibility in the gut of a non-differentiating filter feeder.

In order to investigate this possibility in a mixed algal population the following experiment was performed. Young growing cultures of *Stichococcus, Dunaliella viridis* and a mixture of both algae were divided into two equal portions of 10 ml. each in 50-ml. Erlenmeyer flasks. Into one flask of each pair about 12 *Artemia* larvae were added. The flasks were kept under continuous illumination.

After ten days the following results were recorded: the *Stichococcus* flasks were both equally green, and growing. No appreciable growth of the larvae had occurred although some were still alive. The *Dunaliella viridis* cultures were entirely different. The flask without the animals was bright green, while the flask containing the animals was completely clear, and with the larvae growing well. The results of the mixed cultures were striking. The flask without the animals was deep green and growth of both algae was obvious. (The presence of *Dunaliella viridis* was easily ascertainable without a microscope since motile cells accumulated on the illuminated side of the flask.) The flask with the animals was not as green as the control flask and no obvious population of *Dunaliella viridis* could be seen by superficial observation. The animals in this culture were alive and growing, but they were not as large as the animals grown on the pure *Dunaliella viridis* culture. In the mixed culture without animals microscopic examination revealed the presence of a large number of both *Stichococcus* and *Dunaliella* cells. In the flask containing the animals, very few *Dunaliella* cells could be seen among the many *Stichococcus* cells. Later observations on the flasks, three weeks after the beginning of the experiment, revealed that the well developed *Artemia,* which had eaten and cleared the *Dunaliella viridis,* were dead, apparently of starvation. There was an indication of fresh growth of *Dunaliella viridis.* The animals were also dead in the dense *Stichococcus* sp. culture. On the mixed culture several living individuals were seen. However they were not as well developed as the animals which grow on the pure *Dunaliella viridis* culture.

A similar experiment was performed with a different zooplankton organism, the copepod, *Tigriopus.* The results were similar to those with *Artemia.* These animals could not utilize the *Stichococcus* cells available to them; *Dunaliella viridis* cells, on the other hand, were readily consumed. The ecological implications of these observations are of considerable importance. In standard oceanographic observations all the phytoplankton is considered as available food to the zooplankton. The curious phenomenon, often observed, of the scarcity of zooplankton in waters rich in phytoplankton, and vice-versa, was explained as due either to overgrazing (Harvey *et al.,* 1935) or to animal exclusion by production of inhibitors (Ryther, 1954). The present study suggests the possibility that certain phytoplankton organisms are not suitable food for some planktonic grazers.

SUMMARY

1. Several planktonic algae from the brines of the sea water evaporation ponds were fed to the brine shrimp *Artemia*. They were found to differ in their nutritive value to this filter-feeding animal. One of these algae, *Stichococcus* sp., could not be utilized by the animal as a food source.

2. Controlled experiments of the effect of filter-feeding *Artemia* and *Tigriopus* on a mixed population of two unicellular algae indicate that the animals are capable of acting as differential grazers. The heavy bloom of *Stichococcus* in the evaporation ponds could be due to the effect of preferential digestion of competing algae by grazing animals.

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FURTHER OBSERVATIONS OF HOMING TERNS

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Among the most promising recent experiments in the field of bird orientation have been those of Kramer (1952, 1953) and Matthews (1953a, 1953b) in which pigeons and Manx shearwaters released in unfamiliar territory oriented approximately towards home while still within view of the release point. This ability to choose the homeward direction within a few minutes after release seems to be lost when the sun is obscured by clouds. We have recently reported homing experiments in which common terns (*Sterna hirundo*) showed a directional orientation by tending to fly southeast whether the home direction was northeast, south, or southwest (Griffin and Goldsmith, 1955). In these experiments terns from breeding colonies in Massachusetts and Maine were removed to inland areas, released singly, and observed with binoculars for as long as possible from two points about half a mile apart. The simultaneous cross bearings thus obtained allowed us to reconstruct the first few minutes, and the first one to two miles, of the birds' flight. The terns showed a consistent tendency to head approximately southeast when the sun was visible; but with heavy cloud cover they seemed to scatter at random. The southeasterly headings were independent of the home direction, of the wind direction, of the time of day, and (except for bodies of water) of local topography. Even though common terns do not seem to be as skillful navigators as the pigeons studied by Kramer and Matthews, the results are of interest because they add to the growing body of evidence pointing to the sun as an important factor in the orientation of birds.

Matthews (1955) has questioned whether these results do in fact represent a directional tendency on the part of the terns rather than "a crude form of homeward orientation." We therefore wished to extend our previous observations by adding a release point at which home would lie in the opposite direction from southeast. A further reason for this additional experiment was our suggestion that the habit of flying southeast when suddenly released in unfamiliar inland territory might be an advantageous one for terns nesting along the eastern coast of the United States since it would bring them quickly back to the coast. Both considerations indicated an experiment to compare the initial headings of terns nesting in the Great Lakes region with those from the Atlantic coast.

METHODS

The release point selected for this experiment was the airport at Cortland, New York, about midway between Cape Cod and Detroit. This airport is about 12 miles from the nearest lake of any size (Skaneateles), it has little traffic, and the terrain affords a relatively clear view in all directions. Thirty-two terns were captured late on the afternoon of June 8, 1955 at a nesting colony at Metropolitan

Beach, Michigan (15 miles north of Detroit on the shore of Lake St. Clair); on the morning of June 9, nine others were caught on Penikese Island, Massachusetts. Both groups were transported by automobile in boxes covered so as to prevent the birds from observing their surroundings, and all were released on June 10 between 9:30 A.M. and 6:30 P.M. E.S.T., after 24–48 hours in captivity. The methods of capture and handling were the same as in our previous experiments. The terns were again observed through binoculars mounted on tripods equipped with alidades so that bearings could be noted by an assistant. Three observers participated in this experiment, so that the bearings were taken from the three corners of a triangle with legs approximately 0.4, 0.6, and 0.8 mile in length—the eastern corner being the release point. The terns from the two colonies were released in an irregular sequence, and each bird was set free only after the previous one had been lost from view by all three observers. No attempt was made to check the homing performance by subsequent observations of the nests, since earlier studies had shown that the homing times of terns are too long to contribute any useful information about the route flown (Griffin, 1943).

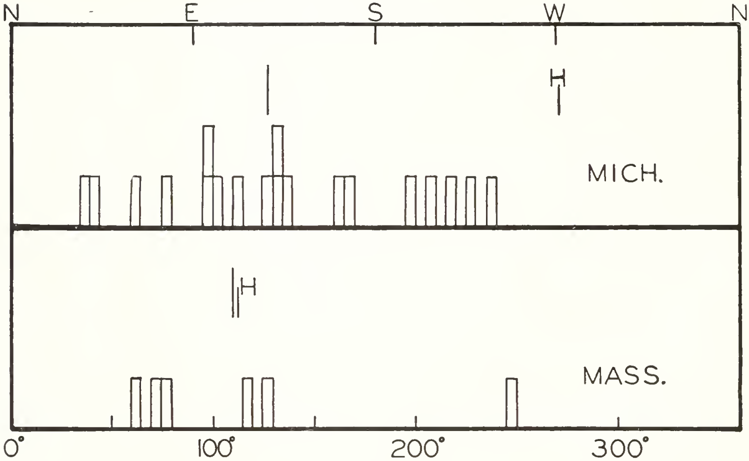

FIGURE 1. Graphic comparison of the initial headings of 25 common terns from colonies in Massachusetts and Michigan released the same day at Cortland, New York. Homeward direction is indicated by an "H", mean heading of each group by an unmarked vertical line.

We should like to express our gratitude to R. Gibbs, W. Jablonski, Mrs. B. Johnston, W. Nickell, A. Novick, R. Payne, and R. Risebrough whose help in capturing and observing the birds made this experiment possible, as well as to the Office of Naval Research which provided financial support through a research contract with Harvard University.

Observations of Initial Headings

All of the terns were released under clear or partly cloudy skies when the position of the sun was evident, though the sun itself was sometimes temporarily hidden behind a cumulus cloud. The day was warm, and there were numerous updrafts on which the terns tended to climb and soar. We thus lost sight of some birds at heights of several hundred feet above the ground before they had indicated a definite heading away from the release point. Unknown to us at the time the releases were begun, there was also a small pond 1.2 miles to the southwest of the release point which attracted some of the terns.

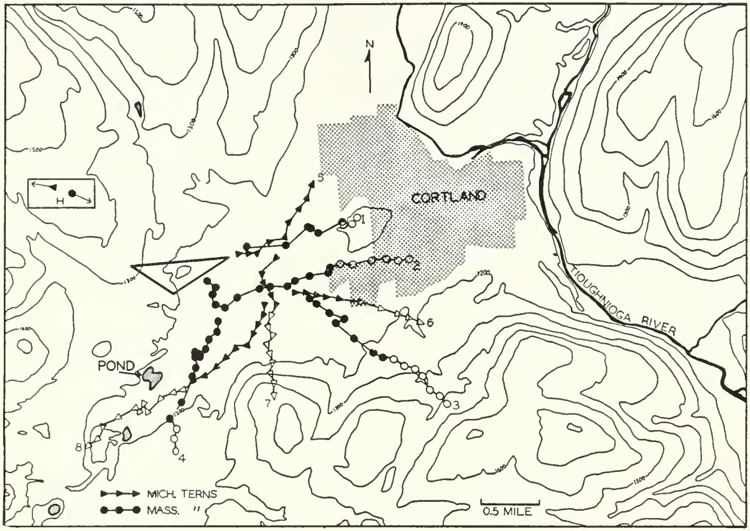

Figure 2. Sample flight paths of eight common terns. Solid circles and triangles stand for crossbearings, open circles and triangles for single bearings. Directions of the home colonies are shown by the arrows in the box (H) at the left. Positions of the observers are represented by the corners of the large triangle. Contour interval is 100 feet. See the text for a discussion.

The results are summarized in Figure 1, in which the points of the compass are "unrolled" on the horizontal axis; each bird is represented by a vertical rectangle. Only those birds are included that gave a distinct heading; the nine attracted to the pond and the seven lost while still circling less than 3000 feet from the release point have been omitted. Home direction is indicated by a capital "H" and a vertical line, the mean heading by an unmarked vertical line. The graph shows that the birds, from the Michigan colony at least, tended to head in a southeasterly direction. The average of the 19 Michigan terns was 139° (40–237°), while the average heading for the 6 Massachusetts terns was 93° (64–130°). In previous experiments with terns from coastal colonies the average initial headings were 140°, 142°, 142°, and 149° when the home directions were 115°, 115°, 44°, and 211°, respectively. The Michigan terns released at Cortland thus showed nearly the same average heading as the coastal terns studied in previous seasons. The more easterly headings of the Massachusetts terns released at Cortland have no obvious explanation, but there were too few birds involved to render the difference between the two groups significant. While the individual headings varied more widely at Cortland than in our previous experiments, the distribution is by no means random, nor do the average headings differ significantly from our previous results.

Figure 2 is a selection of eight sample flight paths plotted from the simultaneous cross bearings and superimposed on a topographic map of the area. The portions of the lines marked by open circles and triangles represent parts of the path for which only one observer had the bird in view and are extrapolations of the earlier part of the flight (solid circles and triangles) for which there were cross bearings. For clarity, some of the first cross bearings from each flight path have been omitted. It is from such flight paths as these that Figure 1 was constructed. Birds numbered 5, 6, 7, and 8 (triangles) are from the Michigan colony, which lies about 335 miles to the west. Numbers 5 and 8 represent the extremes of the distribution; numbers 6 and 7, as well as the other fifteen from Figure 1, lie between these two. Similarly numbers 1, 2, 3, and 4 (circles) are terns from the Massachusetts colony, about 290 miles to the ESE, and numbers 1 and 4 are the extremes of the Massachusetts distribution. This figure shows that the topography had no obvious influence, although certain of the birds (not shown in Figure 2) were attracted to a pond to the southwest. For example, note that number 7 flew over a hill which rises to over 300 feet above the release point while number 2 flew over the city of Cortland. While the birds could certainly see terrain which lies beyond the boundaries of this map, reference to a smaller scale map shows no topographical features which explain the southeast headings.

Discussion

This experiment demonstrates that the tendency to fly in a southeasterly direction when first released in unknown, inland territory is not confined to common terns nesting along the coast; it was equally evident in terns from a colony in the Great Lakes area even though for these birds home lay almost due west. The same directional tendency has thus been observed when the home direction was northeast, east-southeast, south, southwest and west. Austin (1953), who has analyzed the migration route of this species on the basis of banding returns, has shown that both the Great Lakes and New England tern populations have a pronounced southeast component in the fall migration route. But whether this is relevant in the case of birds removed from their nesting grounds during the breeding season is open to question.

Arnould-Taylor and Malewski (1955) have recently suggested that topographic cues have been responsible for most of the results obtained by observing initial headings of homing birds. That topography may be the dominating influence under certain conditions has been firmly established; for example, Griffin (1952) reported that in a series of airplane observations of homing pigeons, certain of the birds followed roads, railroads, and lake shores. We have pointed out that in these experiments with common terns, local bodies of water attract many birds; but, when precautions are taken to avoid release points with ponds in the vicinity, the terns tend to fly approximately southeast. Because it is so difficult to explain such observations on the basis of topographic cues, it is perhaps too soon to abandon all thought that birds employ some more refined method of navigation.

SUMMARY

1. Our observations of the initial flight directions of common terns released in unfamiliar territory have been extended to include birds from the Great Lakes region as well as from the New England coast. When terns from both populations were released on the same day at Cortland, New York, both groups showed a tendency to head approximately southeast.

2. The first mile or two of the terns' flight paths were plotted on a topographic map of the area. Aside from the fact that a small pond attracted some of the birds, topography did not offer any apparent explanation of their headings, and a few persisted on a southeasterly course over moderately high hills.

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MODIFICATION OF X-RAY INJURY TO HYDRA LITTORALIS[1] BY POST-IRRADIATION TREATMENT WITH MAGNESIUM SULFATE AND GLUTATHIONE[2]

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Very few studies have been reported on the damaging effects of ionizing radiations on *Hydra*. Zawarsin (1929), Strelin (1929) and Evlakhova (1946), however, studied the effect of sublethal doses of x-rays and found that inhibition of budding and regeneration varied with the dose of radiation used. Daniel and Park (1951, 1953) reported a toxic effect of x-ray-treated media on *Hydra* tentacles and (1954) direct x-ray damage leading to death in 24 hours.

A number of investigators (Barron *et al.*, 1949; Bellack and Krebs, 1951; Chapman and Cronkite, 1950; Chapman *et al.*, 1950; Patt *et al.*, 1950; Bacq, 1951; Cronkite *et al.*, 1951) demonstrated that glutathione modifies some of the biological effects of ionizing radiations. In general, protection resulted only if the glutathione was given before irradiation. Similarly, in most cases cysteine has to be present at the time of irradiation in order to exert a protective effect (see Patt, 1953). Barron and co-workers, however, found that when glutathione was added to aqueous solutions of succinoxidase after irradiation, the enzyme was partially reactivated. Patt *et al.* (1952) reported protection to mammalian thymocytes when cysteine was added immediately after irradiation.

Daniel and Park (1954) showed that when hydras given 25,000 r were placed immediately in a dilute solution of salts containing either $MgSO_4$ or $MgCl_2$, about twice as many survived 24 hours as were living in the same salt solution without Mg^{++}. In view of this result, and of the few cases reporting modification of x-ray damage by post-irradiation treatment with sulfhydryl compounds, the present studies were made on the effects of continuous post-irradiation treatment with $MgSO_4$ plus glutathione on survival and on budding of hydras.

MATERIALS AND METHODS

The hydras used in the present studies were from a clone culture grown in the laboratory at a room temperature of 25° ± 1.5° C. The cultures were kept in a standard salt solution of 1.7×10^{-3} *M* NaCl, 5.4×10^{-5} *M* KCl and 3.3×10^{-4} *M* $CaCl_2$ in double-distilled water (the second distillation being from glass). This solution contained the same salts and in approximately the same concentration as that used by Daniel and Park (1954), and will hereafter be referred to as "standard saline." The hydras were fed newly hatched brine shrimp daily and were washed and changed to fresh standard saline one hour after each feeding. They were transferred to clean dishes once a week. Under these conditions the hydras reproduced asexually by budding.

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[1] Kindly identified by Dr. Libbie H. Hyman, American Museum of Natural History, New York, N. Y.

[2] These data are from a thesis submitted to the Graduate Council of the George Washington University by Helen D. Park in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

For all experiments, hydras of equal size, without buds, were selected from the stock cultures before the daily feeding and washed in standard saline before treatment. The hydras were irradiated in a Pyrex glass dish containing 50 ml. of standard saline which gave a solution depth of 17 mm. The hydras immediately were washed with standard saline, then within 10 minutes were placed in the solutions to be studied. Except while the hydras were under observation the dishes were kept in moist chambers.

Irradiation factors were 50 kv constant potential, 50 ma beryllium window tube; 2700 r per minute. Dose determinations were made by the method of Andrews and Shore (1950). An aluminum plate 0.020 inch thick served as an x-ray filter and as a dish cover. Water cooling of the irradiation dish kept the temperature of the contents within 2° C. of the temperature of the laboratory.

RESULTS

Survival experiments

In order to compare the effect of glutathione with that of $MgSO_4$, equal numbers of hydras exposed to 25,000 r were placed in (1) standard saline; and (2) 5.0×10^{-4} *M* $MgSO_4$, (3) 1.0×10^{-5} *M* glutathione, and (4) 5.0×10^{-4} *M* $MgSO_4$ plus 1.0×10^{-5} *M* glutathione, each in standard saline. Non-irradiated controls were also treated with the four solutions. The $MgSO_4$ concentration was within the range previously found by Daniel and Park to protect hydras exposed to 25,000 r. Within this range the protective effect of $MgSO_4$ was not a function of the ionic strength of the solutions. The concentration of glutathione had previously been shown to modify a toxic effect of irradiated water on hydra tentacles. The animals were left in their respective solutions 24 hours, at which time the survivors were counted. Five complete experiments, each consisting of 10 animals per group, were carried out in a period of 30 days.

TABLE I

Effect of post-irradiation exposure to $MgSO_4$ and glutathione on survival of hydras after 25,000 r. Fifty hydras in each treatment group

Treatment	Number alive after 24 hours	
	Irradiated	Non-irradiated
Standard saline	12±2	50
$5.0 \times 10^{-4}M$ $MgSO_4$ in standard saline	30±4	50
$1.0 \times 10^{-5}M$ glutathione in standard saline	18±4	50
$5.0 \times 10^{-4}M$ $MgSO_4 + 1.0 \times 10^{-5}$ *M* glutathione in standard saline	21±4	50

Standard error estimated from variation among 5 experiments.

As shown in Table I none of the non-irradiated hydras died. All of the irradiated groups showed by chi square test significantly[3] fewer survivors than their controls. The only statistically significant differences among the irradiated groups were between the hydras in $MgSO_4$ and those in saline, and between those in $MgSO_4$ and those in glutathione. There is not sufficient statistical evidence to assert definitely that either glutathione or the combined treatment had a protective effect against the radiation. The present results confirm the conclusion of Daniel and Park that $MgSO_4$ had a specific protective effect against the radiation.

Budding experiments

Hydras reproduce asexually by the formation of buds which constrict from the parent as adult hydras. The process involves increase in the mass of protoplasm, cell division and differentiation. In the stock cultures maintained in this laboratory, the development of a bud, from the time it is first recognizable until its separation from the parent, takes from two to four days.

The effects of continuous post-irradiation exposure to $MgSO_4$ and glutathione on budding were studied using 4500 r, a dose the author had previously found to be approximately one-third that necessary to inhibit bud production completely for 10 days. Forty irradiated and 40 non-irradiated hydras in groups of 10 were put in the standard saline, $MgSO_4$ saline, glutathione saline and $MgSO_4$ + glutathione saline solutions previously described. Beginning on the first day after irradiation, the hydras were fed daily. All descendents derived from the original 10 hydras in each group were kept with the parents. Each day for 11 days adults and attached buds were counted as separate individuals and all were transferred to fresh solutions in clean dishes. Five experiments, each including all of the treatment groups, were carried out at intervals over a period of two months.

For all analyses[4] of the data, statistical significance or the lack thereof was determined by comparing an average effect over the five replicate experiments with the variation of this effect among the five experiments.

Figure 1 shows, from days zero through eleven, the average number of adults plus buds present per experiment in each treatment group. Among the non-irradiated hydras, those in $MgSO_4$ and $MgSO_4$ + glutathione produced significantly greater numbers of descendents by the end of 11 days than those in standard saline or glutathione. Since neither of the other two intergroup differences among non-irradiated hydras was significant, it seems probable that during the combined exposure it was $MgSO_4$ which caused the increase in budding.

Comparison of the groups in saline alone shows that 4500 r depressed significantly the budding rate. The data for the irradiated hydras in standard saline suggest that the normal budding rate was regained by day nine, but since a parabola does not fit the points better than a straight line, the break in the curve may be fortuitous.

The budding rate of the irradiated hydras in $MgSO_4$ was not significantly greater than that of their irradiated controls. On the other hand, the irradiated hydras in glutathione produced buds at a significantly greater rate than the irradiated controls. The irradiated hydras in $MgSO_4$ + glutathione produced buds faster than the irradiated controls or the irradiated hydras in $MgSO_4$ or the irradiated hydras in glutathione, and at the same rate as the non-irradiated hydras in standard saline. It can be concluded, therefore, that under the conditions of these experiments, continuous post-irradiation exposure to $MgSO_4$ + glutathione restored the budding rate of irradiated hydras to that of non-irradiated hydras in standard saline.

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[3] The .05 level of probability was used throughout the present work.

[4] The author wishes to thank Mr. Jerome Cornfield of the National Institutes of Health for his help in analyzing the data statistically.

In addition to showing the budding rates of all the groups, Figure 1 shows, on the average, the time at which all 10 of the hydras in each group initiated their first buds (*i.e.*, when 20 adults and buds were present in each group). The data for the individual experiments show that all 50 of the non-irradiated hydras in standard saline initiated their first buds by day six. The total number of irradiated hydras in each treatment group that had initiated their first buds by the time all first buds appeared in the non-irradiated standard saline group was: standard saline 17, $MgSO_4$ 39, glutathione 35, and $MgSO_4$ + glutathione 47. It can be concluded that one of the effects of the radiation was to delay the time of appearance of first buds.

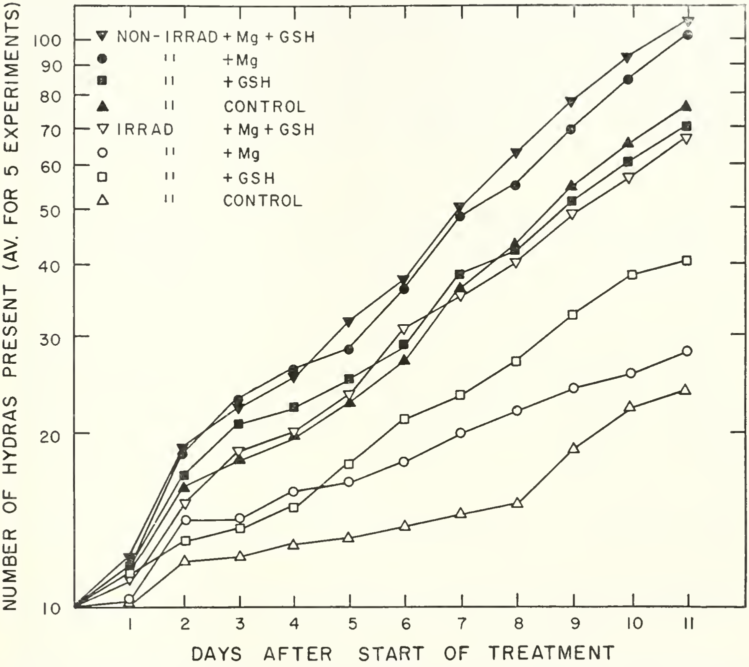

FIGURE 1. Effect of post-irradiation treatment with $MgSO_4$ and glutathione on budding of hydras after 4500 r. Treatment solutions were made in standard saline.

Magnesium sulfate, glutathione, and $MgSO_4$ + glutathione reduced the severity of the radiation effect, but only the combined treatment shortened the time of first bud initiation to that of the non-irradiated, saline controls.

Since the irradiated hydras in $MgSO_4$ + glutathione produced buds faster than the irradiated hydras in $MgSO_4$ alone or glutathione alone, the effect of concentration of the two agents on budding after irradiation was studied in order to ascertain whether the greater effect produced by the two agents together is valid when related to optimal effects of each when used separately. Accordingly, hydras were irradiated with 4500 r and placed in groups of five in solutions in which both $MgSO_4$ and glutathione concentrations were varied from ½ to 16 times those used in the preceding experiments. The hydras were counted on the eleventh day after irradiation.

Table II shows the mean number of hydras present on the eleventh day in each treatment group. The results presented in column 1 show that up to a concentration of 8.0×10^{-3} mole per liter, $MgSO_4$ did not modify the inhibiting effect of 4500 r of x-rays. The results shown in line 1 of the table indicate that glutathione in concentrations between 5.0×10^{-6} and 4.0×10^{-5} mole per liter reduced the inhibitory effect of the radiation on bud production; 1.6×10^{-4} *M* glutathione was highly toxic. Radiation was probably not a factor in this toxicity as five non-irradiated hydras placed in this solution were dead five days later. The data show that the optimal concentrations of the two agents when supplied together were in the range of 2.0×10^{-5} to 4.0×10^{-5} *M* glutathione and 5.0×10^{-4} to 2.0×10^{-3} *M* $MgSO_4$. In addition they show that combined treatment within these ranges resulted in greater bud production than at optimal concentrations of either agent used separately. Furthermore, the amount of budding that took place during exposure to optimal concentrations of both agents together was as great as that of the non-irradiated hydras in standard saline.

TABLE II

Effect of concentration of $MgSO_4$ and glutathione on budding of hydras after 4500 r. Mean number of individuals present on 11th day post-irradiation per 5 hydras treated

Moles per liter of $MgSO_4$	Moles per liter of glutathione						
	0.0	5.0×10^{-6}	1.0×10^{-5}	2.0×10^{-5}	4.0×10^{-5}	8.0×10^{-5}	1.6×10^{-4}
0.0	5.0(5)	6.5(4)	9.0(4)	6.8(4)	10.0(4)	5.0(3)	0.3(3)
2.5×10^{-4}	5.5(4)	13.0(3)	11.3(3)	12.3(3)	11.0(3)	6.0(3)	2.0(3)
5.0×10^{-4}	5.0(5)	9.3(3)	13.0(2)	17.3(3)	19.0(3)	8.3(3)	4.0(3)
1.0×10^{-3}	5.2(4)	9.3(3)	12.0(3)	10.3(3)	13.0(3)		1.7(3)
2.0×10^{-3}	5.4(5)		12.0(3)		22.0(4)	5.0(3)	
4.0×10^{-3}	5.7(3)		10.3(3)		14.0(1)	10.0(3)	1.0(1)
8.0×10^{-3}	5.0(3)		6.0(2)			6.0(2)	4.0(2)

Figures in parentheses = number of groups of hydras treated. The mean number of individuals present on the 11th day in 5 groups of 5 non-irradiated hydras in standard saline was 12.0. No concentration tests were run on non-irradiated hydras.

DISCUSSION

At first glance the effects of $MgSO_4$, glutathione and combined treatment, after the two radiation exposures employed, appear to be anomalous. Since the two sets of results are expressed in different units—number surviving out of total number treated, and rate of increase in numbers of hydras present—they cannot be compared statistically. Taking the apparent discrepancies at face value, however, it seems reasonable that a particular agent might be more effective against a mild cellular damage which would partially inhibit budding than against a more drastic injury leading to death in 24 hours, or that another agent might be more effective in keeping an animal alive for 24 hours than in maintaining it in a reproductive state for a period of 10 days.

The mechanism of the stimulating action of $MgSO_4$ on budding of non-irradiated hydras is not known. However, this effect is perhaps not surprising in view of the fact that $MgSO_4$ has been shown to affect growth in many organisms as widely separated phylogenetically as bacteria (Webb, 1953), protozoa (Mast and Pace, 1939), and mammals (Kruse *et al.,* 1932). Since the addition of increasing amounts of $MgSO_4$ did not increase the amount of budding of irradiated hydras, we may conclude that lack of $MgSO_4$ was not the factor which limited budding after irradiation.

Mechanisms of radiation protection have been considered in reviews by Ord and Stocken (1953) and by Patt (1953). One of the theories of protection by sulfhydryl compounds is that there is a competition by —SH groups for free radicals formed from water in an irradiated solution. Since, in the study reported here, the hydras were not irradiated in the presence of glutathione, and were washed immediately after irradiation and at least ten minutes elapsed between the end of irradiation and beginning treatment with glutathione, the effect on budding would seem to have been due to some mechanism other than a competition of —SH groups for free radicals within or at the surface of the hydra cells.

It is not known whether hydras need an external source of glutathione for budding. If they do, it is possible that the reason glutathione did not stimulate the budding of the non-irradiated hydras was because they were already getting a sufficient amount for budding in their normal intake of food. If hydras do not need an external source of glutathione for budding, stimulation would not occur on the addition of glutathione to the medium.

It was not practicable to determine the amount of food eaten by any of the hydras. However, if the irradiated hydras ate less food than the controls, the rate of budding would be reduced from that of the controls. The effect of glutathione in increasing the budding rate of irradiated hydras might thus have been due to the fact that this agent stimulates mouth opening (Loomis, 1955) which in turn might permit the hydras to consume more food. A second possibility is that the requirement of irradiated hydras for glutathione or some part of the molecule may be greater than that of non-irradiated hydras, *e.g.,* because of the reconstitution of injured regions. Thus the requirement might not be met even with normal food intake, causing a decreased budding rate; addition of glutathione to the medium might satisfy the greater requirement and increase the budding rate over that of the irradiated controls.

The fact that $MgSO_4$ stimulated budding of non-irradiated hydras that were presumably getting an adequate amount of glutathione through their normal intake of food, and the fact that none of the concentrations of $MgSO_4$ used after irradiation had a significantly stimulating effect unless added glutathione was present, suggest the possibility that in all hydras, stimulation of budding by $MgSO_4$ depends on the presence of an adequate level of glutathione or sulfhydryl in the hydra tissues.

SUMMARY

1. Hydras were left for 24 hours in solutions of $MgSO_4$, glutathione and $MgSO_4$ + glutathione after exposure to 25,000 r x-rays. Only the hydras in $MgSO_4$ alone were significantly protected against the effects of the radiation.

2. Hydras were exposed continuously to $MgSO_4$, glutathione, and $MgSO_4$ + glutathione solutions after 4500 r. The rates of budding in each solution were determined. It was found that:

(a) Forty-five hundred r inhibited the budding of hydras significantly.
(b) Magnesium sulfate and $MgSO_4$ + glutathione stimulated budding of non-irradiated hydras while glutathione alone did not.
(c) Magnesium sulfate alone did not significantly modify the inhibitory effect of the radiation on budding.
(d) Glutathione alone partially reversed the inhibitory effects of the x-rays.
(e) Within a fairly wide range of concentrations of $MgSO_4$ and glutathione, the two agents together restored the budding rate of irradiated hydras to that of the non-irradiated animals in standard saline.

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THE MORPHOLOGY AND LIFE–HISTORY OF THE DIGENETIC TREMATODE, AZYGIA SEBAGO WARD, 1910[1]

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The genus *Azygia* was erected by Looss (1899) to contain *Fasciola tereticollis* Rudolphi, 1802 (= *Fasciola lucii* Mueller, 1776, renamed). The worms were from the stomach of *Esox lucius.* According to Dawes (1946), this species, *Azygia lucii* (Mueller, 1776) Lühe, 1909, infects a number of different salmonid fishes, and other species of *Azygia* described from Europe are identical with it. The species has been reported in North America as *Distoma tereticolle* by Leidy (1851) from *Esox reticulatus;* by Stafford (1904) from *Esox lucius, Lota maculosa* and *Ameiurus nigricans;* and as *Azygia lucii* by Cooper (1915) from *Lucius lucius* (= *Esox lucius*), *Lucius masquinongy* (= *Esox masquinongy*), *Lioperca* sp., and immature specimens presumably of the same species were found in *Salvelinus namaycush* and *Micropterus dolomieu.*

Meanwhile, other species of *Azygia* were described in the United States and Canada. Leidy (1851) described *Distoma longum* on the basis of six specimens from the stomach of *Esox estor* Lesueur, 1818 (the American pike), collected near Cleveland, Ohio, and received from Professor Spencer F. Baird. The worms measured 30 to 76 mm. (3 inches) in length and as much as 1.6 mm. in breadth; the maximum diameter of the oral sucker was 1.27 mm. and of the acetabulum 1.06 mm. Measurements given by Leidy for specimens from the stomach of *E. reticulatus,* which he identified as *Distoma tereticolle* Rudolphi, were: length up to 17 mm.; width, 1.06 mm.; oral sucker, 0.52 mm.; and acetabulum 0.7 mm. There is some confusion here since Manter (1926) (p. 66) reported, "Leidy's *Dist. tereticolle* (from *Esox reticulatus*) also was compared with them (specimens of *D. longum* from the Leidy and Cooper collections), and in the single specimen available in the Leidy collection, the oral sucker, contrary to Leidy's description, was found to be slightly larger than the acetabulum." Stafford (1904) erected the genus *Megadistomum* to contain specimens from *Esox masquinongy* which he regarded as identical with *Distoma longum* of Leidy and distinct from *Azygia tereticollis.* Specimens of *Megadistomum longum* (Leidy, 1851) measured up to 5 inches in length when fully extended and up to 3 mm. in breadth, whereas those identified as *A. tereticollis* measured 12 mm. in length and 1 mm. in width. Stafford reported that the largest specimens of *A. tereticollis* were smaller than immature specimens of *M. longum.* Furthermore, he described worms from the stomachs of *Lota maculosa* and of *Stizostedion vitreum* as members of a new genus and species, *Mimodistomum angusticaudum.*

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Marshall and Gilbert (1905) described *Azygia loossi* from the large-mouth bass, *Micropterus salmoides;* the pike, *Lucius lucius;* and the bowfin, *Amia calva.* The worms contained only a few eggs and obviously were not fully mature. They measured 5 to 7 mm. in length, 0.5 mm. in width; the acetabulum was near the middle and the gonads in the caudal one-sixth of the body.

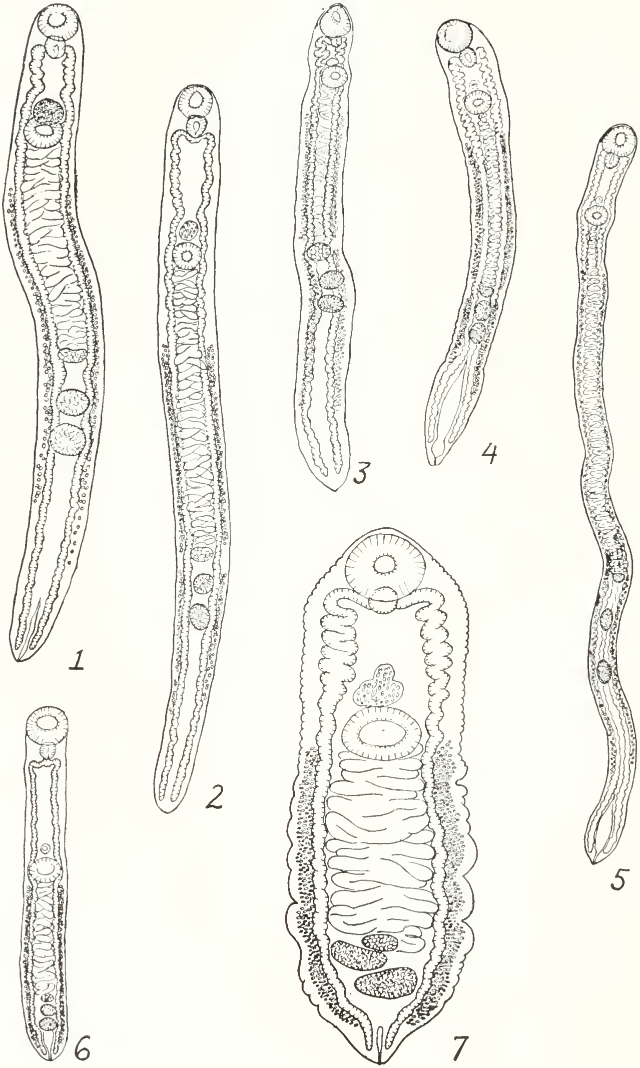
Ward (1910) described *Azygia sebago* from *Salmo sebago* taken at Lake Sebago, Maine. All of seven fishes examined were infected; the worms measured up to 10 mm. in length and from 0.7 to 1.0 mm. in width. From the magnification given, the figured specimen was about 6 mm. long and 0.8 mm. wide. The average diameter of the oral sucker was given as 0.68 mm. and the acetabulum was "distinctly smaller." Specimens presumed to belong to the same species were found in other fishes of Lake Sebago. Two worms were removed from the stomach of a single specimen of *Perca flavescens* and measurements were given for one of them. It was 4.08 mm. long, 0.77 mm. wide; the oral sucker measured 0.51 by 0.57 mm. and the acetabulum 0.35 by 0.40 mm. Four of nine eels, *Anguilla chrysypa* (= *A. rostrata*) were infected with an average of three worms per fish. No descriptive data were given, so presumably they conformed to the specific diagnosis. Eleven of twelve young *Esox reticulatus* were heavily infected; as many as 80 worms were found in a single host. These parasites were more slender and measured 10 to 18 mm. in length. It may be doubted whether they are conspecific with the shorter, more robust worms from the other hosts. Ward reported that smelt, *Osmerus mordax,* were eaten by the larger fishes; the parasites were found also in the stomachs of smelt, although in this host the worms were usually smaller and sexually immature. He noted that the specimens identified by Stafford (1904) as *Azygia tereticolle* are smaller (12 mm. long and 1 mm. wide) than the European species and expressed the belief that they may have been *A. sebago.*

Goldberger (1911) recognized *A. loossi* as a valid species and did not mention *A. sebago,* as Ward's account was probably not available when he wrote his paper. He reported on specimens collected from *Amia calva* taken in Indiana lakes; certain of the worms were identified as *A. lucii,* and others were described as members of two new species, *Azygia bulbosa* and *Azygia acuminata.* Also, he described worms from the stomach of the rock bass, *Ambloplites ruprestris,* as members of a new genus and species, *Hassallius hassalli.*

Odhner (1911) erected the family Azygiidae to contain *Azygia, Otodistomum, Leuceruthrus,* and *Ptychogonimus.* He stated that in the genus *Azygia,* measurements of eggs and extent of vitellaria have little value for specific determination. He declared that *Megadistomum longum* (Leidy, 1851) Stafford, 1904 and *Mimodistomum angusticaudum* Stafford, 1904 are members of the genus *Azygia* and the two generic names were relegated to synonymy. He suggested the probable identity of *A. tereticollis* of America with *A. lucii* of Europe. He criticized Goldberger's work, suppressed *Hassallius* as a synonym of *Azygia,* and expressed the belief that *A. angusticauda, A. loossi, A. acuminata,* and *A. bulbosa* are members of a single species.

As noted, Cooper (1915) described worms which he identified as *A. lucii* from the pike, *Lucius lucius;* the muskellunge, *Lucius masquinongy; Lucioperca* sp.; and immature specimens were recovered from *Salvelinus namaycush* and *Micropterus dolomieu.* He stated that all the worms from the muskellunge are identical with Stafford's *Megadistomum longum* (Leidy) and the smallest one with eggs was 8

Plate I



mm. long. Cooper noted the variable size of worms at the time of egg production. The smallest gravid specimen from the pike was 6 mm. long, but another from the pike, 14 mm. long, was less mature than the one 6 mm. long; others 6 to 14 mm. in length were fully gravid. All the worms from the trout, *S. namaycush,* and the black bass, *M. dolomieu,* including the largest one, 11 mm. long, were immature. Other young and immature specimens from the stomach of *Perca flavescens* were regarded as possible members of this species. Worms from the pickerel (not named) resembled *A. angusticaudum* (Stafford, 1904) but were too contracted to permit positive identification, and others from the pike had a large, globose excretory vesicle, described by Goldberger as characteristic of *A. bulbosa,* but Cooper stated that the shape of the excretory vesicle as well as the length, extent, and "breaking" of the vitellaria are so variable as to be of little use in the delineation of species. Cooper recognized the validity of *A. acuminata,* since 9 specimens from the stomach of *Amia calva* agreed substantially with Goldberger's description of this species.

Ward (1918) stated (p. 392), "Despite many records of its occurrence, the common European *A. lucii* (= *A. tereticolle*) has not been found in North America. Several species peculiar to this continent occur in *Amia calva, Micropterus salmoides* and *dolomieu, Esox lucius* and *reticulatus, Ambloplites ruprestris, Salvelinus namaycush, Lioperca, Lota lota,* and *Salmo sebago."*

Manter (1926) gave a systematic review of the family Azygiidae; he agreed with Ward in regarding the American specimens as specifically distinct from those of Europe but admitted (p. 57) that *"Azygia* is the only genus of the family showing taxonomic confusion in its species." Accepting the statements of Odhner and Ward, he distinguished *Azygia longa* from *A. lucii* on the extent of the vitellaria, which in the European species are reported not to extend behind the testes, and on the shape of the pharynx, which in *A. lucii* is reportedly cylindrical and twice as long as wide. After detailed study and tabular comparison of morphological features, Manter recognized only three species of *Azygia* in North America, *viz., A. longa* (Leidy, 1851), *A. angusticauda* (Stafford, 1904), and *A. acuminata* Goldberger, 1911. Manter confirmed the suspicion of Odhner (1911) that *Azygia loossi* is identical with *Mimodistomum angusticaudum* Stafford, 1904. As syno-

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PLATE I

FIGURE 1. *Azygia lucii,* from *Esox lucius;* specimen collected and identified by Prof. M. Braun, Königsberg, 7 July 1902; 23 mm. long, ventral view; U. S. National Museum, Helminthological Collection No. 3359.

FIGURE 2. *Azygia lucii,* from *Amia calva;* 20 mm. long, ventral view, (Ward Collection) U. S. N. M., Helminth. Coll. No. 51,403.

FIGURE 3. *Azygia longa,* from *Esox reticulatus,* identified by Albert Hassall; 12.4 mm. long; U. S. N. M., Helm. Coll. No. 49.

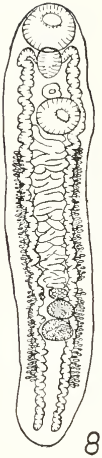
FIGURE 4. *Azygia longa,* from *Esox niger;* 5.2 mm. long, collected 1955 by Paul Krupa, southern New Hampshire.

FIGURE 5. *Azygia longa,* from *Esox niger;* 19 mm. long, collected 1955 by Paul Krupa, southern New Hampshire.

FIGURE 6. *Azygia angusticauda* (type of *Azygia loossi,* Marshall and Gilbert, 1905), from *Micropterus salmoides;* 4.88 mm. long, ventral view; U. S. N. M., Helm. Coll. No. 10,679.

FIGURE 7. *Azygia angusticauda,* from *Stizostedion vitreum;* 10.5 mm. long; ventral view, (Ward Collection) taken by H. W. Manter 4 April 1926, Rock River, Illinois; U. S. N. M., Helm. Coll. No. 51,402.

PLATE II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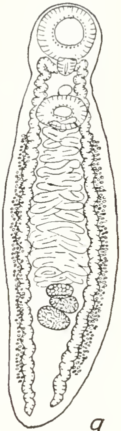
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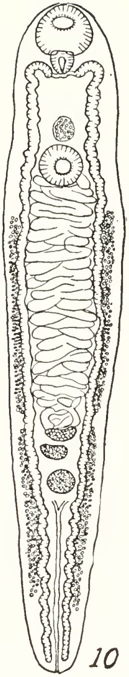
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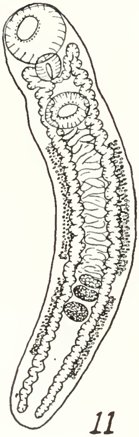
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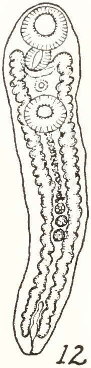
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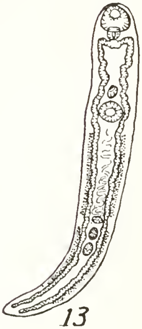
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nyms of *A. longa* (Leidy), Manter listed: *Distomum longum* Leidy, 1851; *Distomum tereticolle* of Leidy, 1851; *Megadistomum longum* (Leidy) of Stafford, 1904; *Azygia tereticolle* of Stafford, 1904; *Azygia sebago* Ward, 1910; *Azygia bulbosa* Goldberger, 1911; *Hassallius hassalli* Goldberger, 1911; and *Azygia lucii* of Cooper, 1915. He discussed the problems of specific determination, noted the bundles of longitudinal muscles which traverse the parenchyma and quoted Leuckart's description of them, and stated that in such elongate and powerfully muscled trematodes, contractions not only alter the general shape of body but the form and relative position of internal organs. Concerning differences in size and sexual maturity, he observed that in the related species, *Otodistomum cestoides,* specimens increase six to seven times in size after attainment of sexual maturity. This fact was used to justify the inclusion in a single species, *A. longa,* of gravid specimens 3.9 mm. long which had been described as *A. bulbosa,* and others which measured up to 3 inches in length and had been described as *A. longum.* It is true that these specimens were from different host species and worms grow larger in larger hosts, but it is doubtful whether host influences can produce such extreme range in size within a single species. Manter's description of *A. longa* was based largely on worms which Ward had described as *A. sebago* and which Manter regarded as identical with *A. longa.* The specific features of *A. sebago* were not clearly defined; there is uncertainty concerning the species, since there is strong probability that material of more than one species was included in the specific diagnosis. According to Manter who studied the Ward collection (p. 64), "*A. sebago* averages about 6 to 8 mm. in length. Specimens were found as small as 1 mm. and no ova were present in forms 2.85 mm. long. . . . Of the other *Azygia* species, *A. bulbosa* Goldberger is most evidently identical with *A. sebago.* Type material of both species was studied. . . . The original type material of *Hassallius hassalli* was also examined for comparison. . . . In fact, after allowance is made for body contraction, this form can not be distinguished from the other common American forms as represented by *A. sebago* and *A. bulbosa.*"

Van Cleave and Mueller (1934) remarked on the variability in fundamental characters, such as the anterior and posterior limits of the vitellaria and the position of the gonads, in the genus *Azygia.* They endorsed the action of Manter in reducing the number of species in North America and went even further in reducing *A. acuminata* to synonymy with *A. longa.* They noted that Manter had listed *A. bulbosa* as a synonym of *A. longa*, and since they regarded *A. acuminata* and *A.*

PLATE II

FIGURE 8. *Azygia sebago,* from *Perca flavescens,* Sebago Lake, Maine, 1907, 4.26 mm. long, ventral view, (Ward Collection); U. S. N. M., Helm. Coll. No. 51,401.

FIGURE 9. *Azygia acuminata,* from *Amia calva,* Indiana, type of Goldberger, 1911, 6.6 mm. long, ventral view; U. S. N. M., Helm. Coll. No. 10,500.

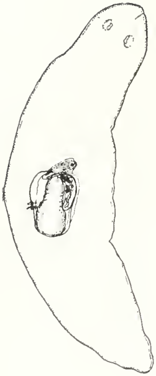
FIGURE 10. *Azygia sebago,* from *Anguilla rostrata,* Falmouth, Mass., 1955, flattened specimen, 12.5 mm. long, ventral view.

FIGURE 11. *Azygia bulbosa,* from *Amia calva,* Indiana, type of Goldberger, 1911, 8.6 mm. long, ventral view; U. S. N. M., Helm. Coll. No. 10502.

FIGURE 12. *Azygia sebago,* from *Anguilla rostrata,* Falmouth, Mass., 1954, immature specimen, 2.66 mm. long, ventral view.

FIGURE 13. *Azygia sebago,* from *Anguilla rostrata,* Falmouth, Mass., 1955, young specimen with 46 eggs in the initial one-half of the uterus, 5.3 mm. long, ventral view.

PLATE III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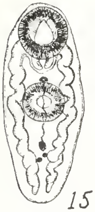
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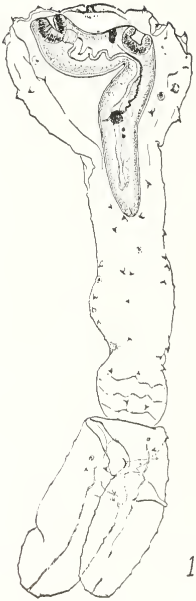
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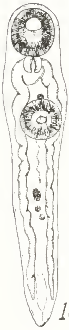
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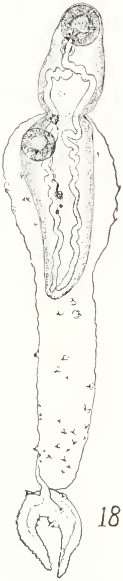
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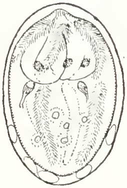
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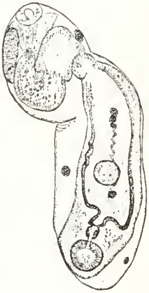
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bulbosa as synonyms, *A. acuminata* should also become a synonym of *A. longa*. The reasoning is sound if the postulates are correct, which now appears doubtful. The specimen shown in their Figure 9 (5) which is referred to *A. angusticauda* and the one Figure 9 (7) referred to *A. longa* are so similar that they are probably conspecific and they may not belong to either *A. angusticauda* or *A. longa*. They closely resemble the worms from the eel, identified in this paper as *A. sebago*.

In other surveys of trematode parasites of fishes, *A. angusticauda* and *A. longa* have been reported in eastern North America but *A. longa* may not extend into the area of Lake Huron and northern Wisconsin and neither species has been found in the fishes of western Canada. Lyster (1939) reported *A. longa* from *Esox lucius* and *Anguilla rostrata* in Canada. The single worm from *E. lucius* is probably a young specimen of *A. longa*, but those from eels are very different. He stated that some of them could be assigned to *A. angusticauda* and specimen No. 1 in his table, which is 4.8 mm. wide at the acetabulum, is probably *A. angusticauda*. The others, which are the same length as the one from *E. lucius* but are twice as wide, are very similar to those from eels on Cape Cod. Miller (1940) reported *A. angusticauda* from *Stizostedion vitreum* and *Micropterus dolomieu* in the central St. Lawrence watershed. Miller (1941) restudied the collection of Stafford. He found a specimen from the muskellunge which he identified as *Megadistomum longum;* it was 18.5 mm. long, 1.2 mm. wide, and there are no eggs in the uterus. Another specimen, from *Lota maculosa* and identified as *A. tereticolle,* is 6.5 mm. long, 0.5 mm. wide, and contains eggs. If these worms belong to *A. longa,* as stated, it is difficult to explain the sexual maturity of the smaller individual. In the Stafford collection Miller found two mature and several juvenile specimens of *Mimodistomum angusticaudum*. One of the mature specimens, 7.25 mm. long and 1.65 mm. wide, shown in his Figure 13, is typical, with the acetabulum near the middle and the gonads in the posterior one-sixth of the body. This account of the original Stafford specimens definitely relegates *Azygia loossi* Marshall and Gilbert, 1905 to synonymy with *A. angusticauda* (Stafford, 1904). Choquette (1951) reported both *A. longa* and *A. angusticauda* from the muskellunge, *Esox m. masquinongy*, in the St. Lawrence watershed. Meanwhile, Bangham (1944) examined 1,330 fishes, representing 38 different species, from 40 different locations in northern Wisconsin. He did not find *A. longa,* but *A. angusticauda* was present in 12 species of fish. Bangham and Venard (1946) examined 676 fishes, belonging to 22 species, from Algonquin Park lakes. Worms from *Anguilla rostrata* were

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PLATE III

FIGURE 14. *Dugesia tigrinum,* 7 mm. long, experimental infection, two juvenile *Azygia sebago* in the pharyngeal pockets.

FIGURE 15. *Azygia sebago,* 0.81 mm. long, natural infection, from pharyngeal cavity of *D. tigrinum,* juvenile worm found by J. Louis Bouchard.

FIGURE 16. *Azygia sebago,* juvenile worm from *D. tigrinum,* 1.17 mm. long, natural infection, specimen from J. Louis Bouchard.

FIGURE 17. *Azygia sebago,* cercaria, naturally emerged, a fixed and stained specimen.

FIGURE 18. *Azygia sebago,* cercaria, from a crushed snail, larva not entirely mature and only partially enclosed in the enlarged, basal end of the tail, furci shriveled, a fixed and stained specimen.

FIGURE 19. *Azygia sebago,* miracidium in egg, from sketches made of living larvae.

FIGURE 20. *Azygia sebago,* redia in which the pharynx is recognizable; the body of the cercaria is 0.8 mm. long, the furci 0.18 mm. long; fixed and stained specimen.

identified as *A. longa;* others from *Micropterus dolomieu, Perca flavescens* and *Lepomis gibbosus* were identified as *A. angusticauda.* Bangham and Adams (1954) did not find *Azygia* in the examination of 5456 fishes, belonging to 36 different species, taken in the Columbia, Fraser and other rivers of western Canada. In a survey of parasites from 1667 fishes, representing 53 species, from Lake Huron and Manitoulin Island, Bangham (1955) found *A. angusticauda* in the northern channel catfish, *Ictalurus l. lacustris.* This parasite obviously can infect a large number of species of fish.

Knowledge of the life-history of azygiid trematodes dates from the publication by Szidat (1932) on the developmental cycle of *Azygia lucii,* a common parasite in the stomachs of salmonid fishes, especially species of *Esox,* in Europe. Szidat found that the large, furcocercous, cystocercous larva, *Cercaria mirabilis* Braun from *Lymnaea palustris,* when fed to young pike, *Esox lucius,* developed in ten days into adult *Azygia lucii.* He recalled the statement of Looss (1894), that when small pike are eaten by larger ones, the azygiid parasites leave the stomach of the ingested fish and establish themselves on the stomach of the preditor; and stated (p. 501), "Überdies sind ältere Hechte keine Planktonfresser mehr, so dass für sie die Übertragung auf dem zuletzt geschilderten Wege den vorherrschenden Modus darstellen wird, und die jugendlichen Hechte demnach biologisch doch als Zwischen- oder Hilfswirte zu werten sind." Szidat reported that other small fishes also ingest the cercariae and may serve as transport hosts, but in these species the parasites do not develop to sexual maturity. He found juvenile *A. lucii* in the stomachs of small predacious fishes belonging to the genera *Perca, Lucioperca,* and *Gasterosteus.* Szidat described the cercaria-producing generation as a redia, which lacks a digestive tract but in which the pharynx persists as an organ for ingesting fragments of the digestive gland of the snail host and also as a birth pore. He traced the development of the cercariae and noted their resemblance to those of the strigeids and schistosomes. The cercariae are not encysted in the snail host. The body of the cercaria sits in a narrow depression at the anterior end of the flattened tail-stem. The cercariae mature in the haemocoele of the snail and emerge into the mantle cavity. In water, the proximal portion of the tail begins to swell and the body of the larva, anchored in the base of the depression by the tubule of the excretory system, is enveloped by the base of the tail and enclosed in it. Szidat also described *Cercaria splendens,* believed to represent a second species of *Azygia,* but the adult stage and final hosts were not discovered.

The achievement of Szidat in working out the life-cycle of *A. lucii* disclosed that the furcocercous, cystocercous larvae of the Mirabilis type, originally regarded by Leuckart as free-swimming sporocysts and shown by Braun (1891) to be cercariae when he described *Cercaria mirabilis,* are developmental stages of azygiid trematodes. The first member of the group was found by Wright in a fresh-water aquarium and described (1885) as a free-swimming sporocyst. Ward (1916) named the species *Cercaria wrighti* and described a second species, *Cercaria anchoroides,* collected in top and bottom tow every day from July 25 to August 5, 1893, in Lake St. Clair, Michigan. Subsequent investigators have reported other members of the Mirabilis group; sixteen species have been described, but some of them are identical. Several of the named species were described from immature stages, taken from crushed snails, and can not be identified with certainty. Others were described from free-swimming cercariae and the hosts are unknown. Certain of them have proved to be larvae of species in the genus *Proteromctra,* erected by Horsfall (1933) to contain *Cercaria macrostoma* Faust, 1918. Reviews of the cystocercous cercariae were published by Horsfall (1934), Smith (1936) and Dickerman (1946). Those with forked tails were designated as furcocystocercous by Le Zotte (1954) who showed that members of the family Bivesiculidae also have larvae of this type.

The second report on the life-cycle of azygiid trematodes was given by Stunkard (1950). Larval distomes had been referred to him for identification in the winter of 1949–1950 by Mr. J. Louis Bouchard, then a graduate student at the University of Oklahoma. The worms had been found in planarians, *Dugesia tigrinum,* received from the Marine Biological Laboratory, Woods Hole, Massachusetts. The structure of the larvae indicated that they were azygiids and study of the life-cycle was begun at the Marine Biological Laboratory in the summer of 1950. Records of the Supply Department of the M. B. L. showed that the planarians sent to the University of Oklahoma had been collected in Morse's Pond in Falmouth. Thirty-eight *D. tigrinum* were collected there on July 10, 1950 and a larval trematode, identical with the specimens sent by Mr. Bouchard, was found in the pharyngeal pockets of two of them. Eight additional worms of natural infection were found in 120 *D. tigrinum* examined. To discover the first intermediate host, different species of mollusks were collected from Morse's Pond and isolated. Furcocercous cercariae of the azygiid type emerged from nine of 246 *Amnicola limosa.* Four planarians, examined under the microscope and known to be uninfected, were placed in a finger-bowl with three specimens of *A. limosa* which were shedding these cercariae. After six days exposure, one to four larvae were found in the pharyngeal cavities of each of the planarians. These larvae were identical with those sent by Mr. Bouchard. The tails, in the bases of which the bodies of the cercariae formerly were enclosed, had completely disappeared. Other planarians were subsequently placed in dishes with infected *A. limosa* and larvae found in their pharyngeal pockets. The larvae may persist for several weeks in *D. tigrinum,* but they do not encyst or grow and it is apparent that the planarians serve merely as paratenic or transport hosts. Attempts to feed the cercariae to goldfish and small perch were not successful; the fish would not take the larvae and when introduced into their mouths, the cercariae were expelled. Planarians infected with cercariae were fed, but the results were uncertain and the short period in which the work could be conducted, led to no further information at that time. It was clear that the larvae belonged to a species of *Azygia,* but specific determination could not be established.

Sillman (1953a) reported that in the vicinity of Ann Arbor, Michigan, the mud pickerel, *Esox vermiculatus,* and the bowfin, *Amia calva,* harbor *Azygia longa.* Eggs of the trematode, containing mature miracidia, were fed to both wild and laboratory-raised *Amnicola limosa.* Cercariae producing rediae were found after 21 days and cercariae emerged 42 days after infection. Cercariae fed to *Esox vermiculatus* developed in 20–30 days into egg-bearing worms. Two of 13,500 *Amnicola limosa* were found naturally infected with cercariae which appeared identical with those in experimentally infected snails.

In a thesis submitted for the Ph.D. degree at the University of Michigan, Sillman (1953b) gave further information. He stated that two species of *Azygia* are present in the Ann Arbor area. One species, which he identified as *A. longa,* occurs in both *Esox vermiculatus* and *Amia calva.* The other species, which he identified as *A. acuminata*, was found only in *Amia calva*. Worms assigned to *A. longa* were somewhat longer, more slender and the suckers were slightly smaller than those of *A. acuminata*, but the measurements of worms and organs overlapped. According to Sillman, the collecting ducts of the excretory system branch from the vesicle behind the testes in *A. longa* and between the testes in *A. acuminata*. Although there was much variation, the average size of eggs in *A. longa* was 55 by 31 microns whereas that of *A. acuminata* was 69 by 38 microns. Furthermore, specimens of *Amnicola limosa* did not become infected when fed eggs of *A. acuminata*.

Investigation of the life-cycle and development of *Azygia* has been continued at the Marine Biological Laboratory, Woods Hole, Mass., during the summer months since 1950. An abstract of the results was presented (Stunkard, 1955). Infected snails were found each year and the morphology of the young distome, especially the details of the excretory system, was studied. Hundreds of fishes, including *Esox niger, Perca flavescens, Morone americanus, Micropterus salmoides, Micropterus dolomieu*, and others, were examined in the attempt to find the sexually mature stage of the parasite. The first to be discovered, a small, immature specimen of *Azygia* (Fig. 12) was found in the stomach of an eel, *Anguilla rostrata*, late in the summer of 1954. During the summer of 1955, 42 eels were examined; 10 of them were infected and many fully mature worms were collected. Continued examination of other fishes, especially the pickerel, *Esox niger*, from the same ponds where the infected eels were taken, has not disclosed infection by members of the genus *Azygia*, and it appears that the eel is the natural and possibly the only host for the species in the Woods Hole region. The larger ponds in the area are under the control of the Division of Fisheries and Game, Bureau of Wildlife Research and Management of the State of Massachusetts, and many of them have been stocked with game fishes from time to time. Through the kind cooperation of Mr. Russell Cookingham, a large number of fishes, belonging to various species, were provided during the summer of 1955, when certain of these ponds were inspected to determine their productivity.

Specific determination of the parasites from the eel has proved difficult. Descriptions are wholly unsatisfactory and accordingly, specimens of *Azygia* in the U. S. National Museum were borrowed through the kindness of Dr. E. W. Price and Mr. Allen McIntosh. The material consisted of 6 specimens in alcohol (bottle M 248-D), the type specimens of *Distomum longum* Leidy, and other specimens mounted on slides and bearing the following labels, U. S. National Museum, Helminthological Collection:

No. 49. *Distomum longum* from *Esox reticulatus*, determined by Albert Hassall; 1 slide. (Plate I, Fig. 3.)
No. 3359. *Azygia lucii* from *Esox lucius*, collected and determined by Professor M. Braun, 7 July 1902, Königsberg, Germany; 1 slide. (Plate I, Fig. 1.)
No. 10500. *Azygia acuminata* from *Amia calva*, type and paratypes; 4 slides. (Plate II, Fig. 9.)
No. 10502. *Azygia bulbosa* from *Amia calva*, type and paratypes; 3 slides. (Plate II, Fig. 11.)
No. 10679. *Azygia loossi* from *Micropterus salmoides*, cotypes; 3 slides. (Plate I, Fig. 6.)

No. 51399. *Azygia sebago* from *Salmo sebago,* 5.2 mm. long, 1 slide, H. B. Ward collection.

No. 51401. *Azygia sebago* from *Perca flavescens,* H. B. Ward collection; 2 slides. (Plate II, Fig. 8.)

No. 51403. *Azygia sebago* from *Amia calva,* 20 mm. long, 1 slide, H. B. Ward collection. (Plate I, Fig. 2.)

No. 51402. *Azygia angusticauda* from *Stizostedion vitreum,* collected by H. W. Manter, 4 April 1926, Rock River, Illinois, 2 slides, H. B. Ward collection. (Plate I, Fig. 7.)

Examination of the specimen of *Azygia lucii,* No. 3359 in the U. S. National Museum, invalidates the criteria used by Ward and Manter to distinguish between *A. longa* and *A. lucii.* In this specimen (Fig. 1) which measures 23 mm. in length, collected and identified by Professor M. Braun, the pharynx is not twice as long as broad; in fact, the organ measures 0.80 mm. long and 0.60 mm. wide. Furthermore, the vitellaria extend far behind the posterior testis; the follicles on the left side about one-half the distance from the testis to the end of the body. In the Ward collection there is a specimen from *Amia calva* (Fig. 2) which measures 20 mm. in length and which resembles the European specimen so closely that I am disposed to regard the two as specifically identical. Leidy, Stafford and Cooper all reported the finding of *A. lucii* and it appears that this species does occur in North America. *Esox lucius,* the type host, is circumpolar in range, and the distribution of its parasites may be expected to parallel that of the host. The dispersal of fishes in the northern hemisphere following the last glacial period has been traced by Walters (1955).

Although the criteria used by Ward and Manter to distinguish *A. longa* from *A. lucii* are inadequate, the two forms are probably distinct. About 100 specimens collected by Mr. Paul Krupa from *Esox niger* in southern New Hampshire during the summer of 1955 are so similar to the six worms in alcohol, now in the U. S. National Museum, which constitute the original material of the species described by Leidy (1851) as *D. longum,* that they must be regarded as identical. A representative example from the Krupa collection is shown (Fig. 5) and a smaller one (Fig. 4). These worms are very slender. The Krupa specimens were dropped in cold Duboscq-Brasil fluid and fixed without narcotization or pressure. Oviferous specimens vary from 4 to 26 mm. in length and 1.1 mm. is the greatest width. The width does not increase very much as the worms grow in length. Comparison of Figure 5 with that of *A. lucii* (Fig. 1) portrays what are believed to be specific differences. Further evidence that *A. longa* is distinct from *A. lucii* is afforded by comparison of the cercariae. *Cercaria mirabilis* Braun, 1891, shown by Szidat (1932) to be the larval stage of *A. lucii,* is very different from the cercaria described by Sillman as the larval stage of *A. longa.* Moreover, in Europe *A. lucii* uses a pulmonate snail, *Lymnaea palustris corvus,* as the first intermediate host, whereas according to Sillman, the asexual stages of *A. longa* occur in the pectinibranchiate snail, *Amnicola limosa.*

Recognition of two distinct species, *A. lucii* and *A. longa,* may resolve certain difficulties and clear up confusion in the literature. The worms from *Esox masquinongy* which Stafford (1904) described as *Megadistomum longum* (Leidy) measured up to five inches in length when extended and probably were not identical with *A. longa* of Leidy. Stafford reported a specimen 18 mm. long which contained no eggs. Cooper (1915) identified specimens from *E. masquinongy,* which he regarded as identical with those of Stafford, and others from *E. lucius,* as *Azygia lucii.* His specimens from the muskellunge measured 21 to 48 mm. in length and 1.40 to 2.40 mm. in width, whereas those from the pike were 14 to 20 mm. in length and 0.74 to 1.42 mm. in width. Comparison of the small worms from *E. masquinongy* with worms from *E. lucius* led Cooper to regard them as conspecific. But he was unable to account for the variable size at which eggs are produced in different individuals. He reported that a specimen 14 mm. long from *E. lucius* was less mature than another 6 mm. long from the same host species, and that worms from the trout and small-mouthed black bass were all immature although one from *S. namaycush* was 11 mm. long. Discussing the effect of season on sexual maturity, Manter (1926) wrote (p. 67.) ". . . it is certain that what is evidently the same species does not attain sexual maturity at the same time in different hosts in which it occurs. Thus, while average sized forms are producing eggs in such hosts as pike, pickerel, and salmon, specimens fully as large are still sexually immature in such hosts as smelt, trout, small mouthed black bass, and perch." Admittedly, members of a trematode species attain a greater size in a larger host species, and *E. masquinongy* is much larger than *E. lucius,* but present information strongly indicates that *A. longa* is distinct from *A. lucii,* if, indeed, the large American species is actually *A. lucii* of European fishes.

All previous authors have agreed on the identity of *A. angusticauda* (Stafford, 1904) and *A. loossi* Marshall and Gilbert, 1905. A cotype specimen of *A. loossi* (U. S. Nat. Mus., 10,679), shown in Figure 6, is 4.88 mm. long and is obviously young, with only a few eggs in the uterus. A fully mature, gravid specimen (U. S. Nat. Mus., No. 51,402) from the walleye, *Stizostedion vitreum,* collected by Manter in 1926, which measures 10.5 mm. in length, is shown in Figure 7. In both, the acetabulum is near the middle and the gonads are situated in the caudal one-sixth of the body. The distinctness of this species appears to be well established.

The specimens of *Azygia* found in the eel at Woods Hole are clearly distinct from *A. angusticauda* and, as noted, are probably distinct from *A. longa.* Specimens of *A. longa* are slender and much elongate; those from the eel are shorter and more robust. The worms collected by Mr. Krupa from *Esox niger* in New Hampshire and identified as *A. longa* remained well extended when dropped into Duboscq-Brasil killing fluid, whereas those from the eel contracted strongly with the result that the length was only 6 to 8 mm., less than one-half that of *A. longa.* Accordingly, most of the worms from the eel were killed and fixed under pressure, which resulted in longer, wider, and flatter specimens. The size of the suckers increased as a result of the compression but comparison of Figures 10 and 13, which were made from one of the largest and one of the smallest oviferous specimens, with Figures 5 and 4, of comparable specimens of *A. longa,* portrays differences between the two forms which are believed to be specific.

Whereas the worms from the eel differ distinctly from those identified as *A. longa,* they agree almost completely with Goldberger's description of *A. acuminata* and agree almost as well with the descriptions of *A. sebago* as given by Ward and Manter. Certain worms from the eel are very similar to specimens in the Ward collection labelled *A. sebago.* It is probable that Ward had more than one species and that his description of *A. sebago* was based on specimens of both *A. longa* and

A. sebago. The worm from the Ward collection which bears the U. S. Nat. Mus., No. 51,401, shown in Figure 8, is clearly *A. sebago,* and the worm on U. S. Nat. Mus., No. 51,403, from *Amia calva* shown in Figure 2, is so like *A. lucii* (*cf.* Fig. 1), that the two might be regarded as specifically identical. Other specimens of *A. sebago* agree so completely with Goldberger's description of *A. acuminata* (compare Figs. 8 and 9), that I am inclined to regard them as identical. Since Ward probably confused two species in his description of *A. sebago*, the removal of the elongate specimens leaves the description virtually the same as that of *A. acuminata.* Specific determination may be impossible on the basis of adult morphology alone and knowledge of life-cycles and larval stages may be required to finally solve the problem. Why the species occurs only in *Anguilla rostrata* in the Woods Hole area is quite unknown. The larval stages are relatively abundant in the snails of the region, but sexually mature worms have so far been found only in the eel. The chain pickerel, *Esox niger,* is common in these ponds where it has been introduced in stocking operations. Since the worms develop in eels in ponds where pickerel, perch, bass and other fishes are not infected, it appears either that the ecological conditions and food-chain lead to the infection of eels rather than other fishes or else the other fishes do not retain the parasites. In the latter event, a separate species must be involved.

When worms were removed from the stomachs of eels and placed in pond water, the eggs in the terminal coils of the uterus were extruded in a string of mucus. These eggs appeared to be fully embryonated and the miracidium was studied in the egg. Although active, the larvae did not emerge in water and hatching occurred only after the eggs were ingested by the snail host. Empty shells were recovered in the feces of *Amnicola limosa* that had eaten the eggs. Some of these snails were found later to be infected but since they had been collected from locations where previous exposure to infection was liable, it would be difficult if not impossible to distinguish between a natural infection acquired before collection and an experimental one. But the snails laid eggs in the finger bowls and young laboratory-raised specimens were fed eggs of the parasite. These small snails became infected and although emerged cercariae were not obtained before the end of the summer, the developmental stages in these experimental infections were indistinguishable from comparable stages in natural infections. In nature, the eggs of the parasite are passed in mucous material from the intestines of eels and settle on vegetation and on the slimy surfaces of submerged rocks and sticks. The snails rasp these surfaces for the diatoms which form a major constituent of their food and incidentally ingest the eggs. The larvae remain alive for long periods and since the eggs do not hatch until they are eaten, the probability of reaching a suitable host and continuing the life-cycle is much enhanced. The larvae emerge in the intestine of the snail and bore through the wall to reach the haemocoele, where they become sporocysts. Young sporocysts have been found adjacent to the intestinal wall two weeks after eggs of the parasite were added to the finger bowl with the young snails. Older infections with rediae and developing cercariae were found later, which definitely link the experimental and natural infections. However, the rate of development of the parasites and the degree of maturity of the infection are not regarded as significant. It is common knowledge that asexual stages of digenetic trematodes persist but fail to grow or reproduce if the hosts are not fed. Thus, infections overwinter in a quiescent stage in mollusks that are dormant or in which metabolism is reduced to a low level. In the present instance, although various methods, including those recommended by Moore *et al.* (1953) and by Sandground and Moore (1955) for the rearing of related snails, were employed, it was obvious that the snails, although most of them remained alive, were not properly nourished, did not grow normally, and the tissues had the atrophic appearance typical of inanition.

Cercariae from natural infections were snapped up by guppies and by small bluegill sunfish, *Lepomis macrochirus*, 2 to 4 cm. in length. The young worms were recovered from the stomachs of these sunfish two and three weeks after they were eaten, but there was very little development of the parasites. These small fishes also ate planarians, *Dugesia tigrinum;* so in nature the fishes could contract the infection by eating either the cercariae or infected planarians. The tails of the cercariae cease to beat after about 48 hours and they would then not be attractive to fishes; moreover the larvae die during the next 48 hours. As stated earlier, the young worms live for weeks in the pharyngeal pockets of *D. tigrinum* and this accessory method of employing an additional paratenic or transfer host enhances the likelihood of survival and aids in the completion of the life-cycle. The cercariae are probably not eaten by eels which are at the end of the food-chain that leads to their infection.

Description of Stages in the Life-Cycle

Adult

The worms are only slightly flattened, almost cylindrical, with rounded ends and enormously developed musculature. Because of the ability to extend and retract the entire body or particular regions to an extraordinary degree, measurements of length and width and location of individual organs have limited significance. A specimen may extend to four or five times its length when contracted, and contraction of different regions can make distances between organs so variable that measurements may be very misleading. Ward (1918) wrote (p. 392), "*Azygia* is a powerfully muscular type and is usually much distorted in the process of preservation so that a lot of specimens taken from the same host at the same time present marked external differences in the preserved condition. Such extreme specimens have been the basis for various new genera, *e.g., Megadistomum* of Leidy and Stafford, *Mimodistomum* of Leidy (*sic*) and *Hassallius* of Goldberger. This same factor has lead to the separation of too many as species." Oviferous specimens from the eel, fixed by the shaking method of Looss, are 3 to 9 mm. long and when fixed under pressure measure 4 to 12.5 mm. in length. Because of the variations caused by muscular contractions on the shape of the body and location of organs, dimensions of the suckers and gonads provide the most reliable morphological data, but these organs appear larger in specimens that have been fixed under heavy pressure. Egg sizes vary too much to provide reliable specific criteria. The worms continue to grow after sexual maturity. A large one and a small one are shown in Figures 10 and 13; both were fixed under pressure and are therefore comparable. Measurements in millimeters of the larger one are: length, 12.5; width, 2.2; oral sucker, 0.96; acetabulum, 0.8; pharynx, 0.36 long and 0.32 wide; ovary, 0.54 by 0.23; anterior testis, 0.5 by 0.33; posterior testis, 0.5 by 0.4. Corresponding measurements of the smaller worm are: length, 5.3; width

0.65; oral sucker, 0.41; acetabulum, 0.34; pharynx, 0.19 long and 0.16 wide; ovary, 0.195 by 0.12; anterior testis, 0.195 by 0.143; posterior testis, 0.24 by 0.16. The eggs, alive, averaged 0.06 by 0.034 mm.; under oil immersion and slight pressure, to study the miracidium, they were slightly larger; in fixed and stained worms they were smaller, and averaged 0.055 by 0.030 mm. In such mounted specimens the eggs are usually collapsed and distorted.

Miracidium

The miracidium of *Azygia lucii* was described by von Nordmann (1832), Schauinsland (1883) and Looss (1894) and that of *Azygia acuminata* (possibly a synonym of *A. sebago*) by Manter (1926). The miracidium of the worms identified as *A. sebago,* studied alive in the egg (Fig. 19) and in stained sections of gravid worms, is similar to that of related genera in the family Azygiidae, as reviewed by Manter (1926). Like the others, it lacks cilia and is provided with bristle plates or plaques. It almost fills the egg-shell; the anterior end may be protruded as a conical papilla on which the ducts of the secretory cells open. Radiating from this area, there are five plates or plaques that bear fine bristles arranged in a chevron-like pattern. The anterior ends of the plates are separated by short intervals, which become wider posteriorly. The plaques extend backward about one-third of the length of the larva; the bristles on the anterior portions are larger and longer than those more posteriad. From a naked area at the posterior end of the larva, four bristle-bearing bands extend forward past the middle of the body. The bands are equidistant from each other and both the anterior and posterior ones manifest a spiral tendency, but this aspect may be the result of rotation of the larva within the shell. The appearance of the miracidium is almost identical with that of *Proterometra macrostoma* as reported by Hussey (1945). Hussey described a structure, designated by earlier authors as a "primitive gut", with four nuclei arranged in a linear series. In *A. sebago,* the corresponding structure, which is glandular and probably serves in penetration, consists of four cells which lie side by side rather than in linear series. These cells are disposed as reported by Manter (1926) for the miracidia of *Otodistomum cestoides, Otodistomum veliporum* and *Azygia acuminata.* Manter reviewed previous accounts and presented a strong argument that the organ is not a primitive gut, but a group of unicellular glands. Immediately posterior to the glandular organ there is a bilobed "brain" and the region behind it contains several large, germinal cells. On either side, near the middle of the body, there is a single flame cell from which an excretory tubule leads caudad, but the ducts were not traced to the pores.

Asexual generations

The youngest sporocyst was recovered from a loose network of connective tissue on the somatic side of the intestinal wall of a laboratory-raised snail that had been exposed 12 days previously. It was oval, 0.094 by 0.062 mm., with no lumen; it contained germinal cells but no germ-balls (embryos). Other larger sporocysts were found in older infections; one, 0.126 by 0.08 mm., contained germinal cells and 6 small germ-balls; another, 0.189 by 0.12 mm., contained germinal cells and 9 germ-balls of varying sizes. In a snail killed one month after exposure, the mother sporocyst could not be recognized but there were 26 rediae scattered about in the haemocoele. The smallest was 0.25 by 0.18 mm., and in addition to germinal cells it had four small spherical to oval germ-balls, 0.02 to 0.04 mm. in diameter. A redia with larger germ-balls but no recognizable cercariae measured 0.57 mm. long and 0.18 mm. wide; the pharynx was 0.08 mm. in diameter and there was a sac-like gut, 0.11 mm. long and 0.032 mm. wide. The largest redia was 1.3 by 0.3 mm. and in addition to smaller embryos, it contained two cercariae, one of which was more than half-grown and had small furci. Whether or not there is a second generation of rediae was not determined.

The cercaria-producing generation of species in the genus *Azygia* was recognized by Szidat (1932) as redial, although the pharynx undergoes reduction to a mere vestige and the intestine completely disintegrates. As noted by Szidat in *A. lucii,* the pharynx, which he termed "rudimentary", serves for the ingestion of bits of the digestive gland of the host and persists as a birth-pore through which the cercariae emerge. The small rediae are vermiform and very active; the pharyngeal end may be inrolled and then everted, while the opposite end may be protruded as a pointed, tail-like structure. Older rediae may extend to a length of 3 mm. and on contraction of the circular muscles, present an annulate appearance. On contraction of the longitudinal muscles they become oval and about 1 mm. in width. The one shown in Figure 20 is bent and as mounted measures 1.12 by 0.325 mm.; in it the pharynx is still distinct. The older, larger, rediae have little mobility but pulsations of one and sometimes two can occasionally be seen through the shell of an infected snail. The number of cercariae in a redia is small; often there is only one and rarely are there more than three recognizable cercariae; other individuals are still in the germ-ball stage, together with a few germinal cells attached to the body wall, chiefly at the posterior end of the redia. Apparently the development of one cercaria restrains the development of others. An infected snail may liberate one or two cercariae each day for a few days and then none for a week or more. The large size of the cercariae is correlated with the slow development and the small number produced.

Cercaria

Developing cercariae are typical furcocercous larvae. As the embryo reaches a length of approximately 0.25 mm., a constriction appears and gradually separates the posterior one-fourth to one-third of the larva as an oval, tail-rudiment. At about this stage, the oral sucker is faintly outlined. When the larva has reached a length of 0.4 to 0.5 mm., the suckers are distinct, the acetabulum is in the posterior half of the body, the tail is about three-eighths of the total length, and the furcal buds are beginning to appear. As development proceeds, the tail increases in length more rapidly than the body; its basal portion, about one-sixth of its length, begins to enlarge and by the time the gonads are recognizable, the anterior end of the tail forms a cup-like ring (Fig. 20), at the base of which the constricted caudal end of the distome is continuous with the tissues of the tail. The cercariae complete their growth in the rediae and emerge into the haemocoele of the snail. While studying the excretory pattern of a redia which was under some pressure, an immature cercaria emerged, tail first, through the old pharyngeal opening. The cercariae mature in the haemal sinuses of the snail, especially the branchial sinus, and emerge through the respiratory opening. During growth, the basal portion of the tail is much enlarged by the accumulation of spongy, fibro-elastic, alveolar tissue which, when the cercaria emerges from the snail, absorbs water and expands rapidly. As a result, this portion of the tail extends forward, encapsulating the body of the cercaria. If infected snails are crushed and immature larvae are liberated into water, the base of the tail is unable to completely engulf the body of the larva (Fig. 18).

Mature, normally emerged cercariae measure 1.8 to 2.3 mm. in length. The expanded, basal portion of the tail is flattened, 0.5 to 0.75 mm. in width, and slightly more in length. The stem of the tail, that portion from the spongy, rigid, basal part to the furci, is 1.0 to 1.5 mm. in length and 0.26 to 0.46 mm. in width. It tapers slightly from the basal to the distal end. It is distinctly flattened and set at right angles to the dorso ventrally flattened body of the larva, so that when looking at the flat aspect of the tail, the body appears in lateral view (Fig. 17). This stem portion of the tail consists of two bands of longitudinal muscles, one on each of the flat surfaces. These muscles are attached at one end to the rigid, spongy portion of the tail and at the other end to the bases of the furci. The furci are flattened, 0.55 to 0.90 mm. in length and 0.20 to 0.28 mm. in width. Normally they are held almost at right angles to the tail stem, whose muscle bands contract alternately, so that the flapping of the tail from side to side produces a sculling effect that pulls the larva through the water. After the beat of the tail is unable to lift the larva from the bottom, it continues for a day or two and this flapping motion makes the larva an attractive lure for small fishes and perhaps other predators. The basal end of the tail becomes sticky and may lightly attach the larva to the substratum. How the larvae reach the pharyngeal cavity of the planarians is not clear. The body is firmly enclosed in the chamber at the anterior end of the tail and could be liberated only by dissolution of the tail. According to Hyman (1951, p. 107), "The triclads do not swallow their food whole but suck it in by peristaltic action of the protruded pharynx." and (p. 199), "The Turbellaria are as a class carnivorous. . . . Favorite items of food of the smaller species are rotifers, copepods, cladocerans, nematodes, annelid worms, etc.,". Perhaps the planarian seizes the larva, and as the tail is sucked in and digested, the young worm is liberated and attaches to the external surface of the pharynx, whence it is carried into the cavity when the pharynx is retracted.

The tail bears many papillae, scattered somewhat irregularly over the surface except for the distal three-fourths of the furci. Each is about 0.05 mm. in diameter, 0.025 mm. tall, and is surmounted by a recurved hook, 0.012 to 0.015 mm. in length. The tail also has many opaque patches, which on higher magnification are seen to consist of minute spherules. The excretory system of the larval body is continuous with that of the tail and the constricted caudal end of the body contains the common excretory canal which traverses the stem of the tail, bifurcates at the bases of the furci, and the resulting tubules open at the tips of the furci. The pattern of flame cells in the tail was not resolved.

The morphology of the young worm, released from the chamber in the tail, is typically azygiid (Figs. 15, 16). The cuticula is unarmed but the preacetabular region bears many papillae and a bristle has been observed at the tip of certain of them. There are at least a dozen papillae, 0.018 to 0.020 mm. in diameter, around the anterior end of the worm. Living specimens vary from 0.7 to 1.3 mm. in length and 0.16 to 0.28 mm. in width. The acetabulum varies from 0.10 to 0.13 mm., and the oral sucker, 0.11 to 0.14 mm., in diameter. The pharynx measures 0.05

to 0.07 mm. in length and usually slightly less in width. The digestive ceca are filled with yellow material, derived from the digestive gland of the snail. The excretory system is complex but has been worked out completely. The pore is terminal and a common duct leads forward almost to the level of the testes. The posterior one-half of this duct may expand to form a bladder-like enlargement, or if the pore is blocked and fluid accumulates, the enlargement may extend farther forward. Behind the testes the common duct divides, forming two ducts which pass forward, median to the digestive ceca. As the ceca turn mediad to join the pharynx, the excretory ducts pass below them and continue on either side of the oral sucker almost to the anterior end of the body. There is, however, no connection between the ducts of the two sides. Anterolateral to the oral sucker, the duct of each side doubles backward and continues posteriad, giving off eleven branches. Each branch divides three times, forming two primary, four secondary and eight tertiary branches. Each tertiary branch receives the capillaries from four flame cells. The flame cell formula accordingly is 2 (11 × 32) or 704 flame cells in the body. This observation is in agreement with that of Looss (1894) who described the same pattern in *Azygia tereticolle* (= *A. lucii*). He regarded the ascending portions of the excretory system as parts of the excretory vesicle and the descending limb with its branches as the collecting ducts. He suggested the possibility of variation in the number of branches and of anastomoses between collecting ducts; however, I have found a constant number of branches and the apparent anastomoses can be resolved as places where one duct crosses another. Counting backward from the anterior end of the body, the first side branch is located at the level of the oral sucker; the second is at the level of the bifurcation of the digestive tract, *i.e.,* the posterior end of the pharynx; the third branch is anterior to the acetabulum; the fourth is at the middle of the acetabulum; the fifth is at the level of the posterior end of the acetabulum; the sixth and seventh are close together a short distance behind the acetabulum; the eighth, ninth and tenth are almost equally spaced; while the eleventh and last, which is the terminal group of the recurrent limb, is distributed to the extreme posterior end of the body around the excretory bladder. The reproductive organs are represented by groups of deeply staining cells, shown in Figures 15, 16 and 17.

SUMMARY

A chronological account of the genus *Azygia* discloses discordant observations and divergent opinions. Dawes (1946) recognized only a single species, *A. lucii,* in Europe. In it he included *A. robusta* Odhner, 1911, which reaches a length of 47 mm. and *Ptychogonimus volgensis* von Linstow, 1907, which measures 5 to 6 mm. in length and had been transferred to *Azygia* as a valid species by Odhner (1911). In America several species have been described, but there is no agreement on the number that are distinct and valid. In fact, there is no adequate information on the extent of variation that occurs in a natural species, and consequently on the features that can be relied on to distinguished between species. This situation is not peculiar to *Azygia,* but obtains in many genera. It is the natural result of development by members of a parasitic species in different hosts, invertebrate and vertebrate, often of different taxonomic groups, which differ in their nutritional and other physiological conditions, and accordingly influence the development and morphological features of the parasite. Until the life-cycle is known and the variation that normally occurs in each possible host is measured, the precise limits of specificity will remain uncertain. Comparison of specimens and descriptions indicates that *A. lucii* may be endemic in North America, that possibly it is distinct from *A. longa* (Leidy), that *A. angusticauda* (Stafford) is a valid species, and that the species described by Goldberger (1911) may be identical with *A. sebago* Ward. Information concerning the life-history of species in the genus *Azygia* is meager. Szidat (1932) showed that *Cercaria mirabilis* Braun is the larva of *A. lucii.* He described a second larva, *Cercaria splendens,* presumably another species of *Azygia,* but the adult stage remains unknown. Sillman (1953a) reported the life-cycle of a species that he identified as *A. longa* and the present paper presents data on the morphology and life-history of a species believed to be *A. sebago.* Stages in the cycle are described and figured.

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TWINNING AND REPRODUCTION OF TWINS IN PELMATO-HYDRA OLIGACTIS

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This paper is written to extend the observations of the writer (Turner, 1950; 1952) on the reproductive potential in clones of *Pelmatohydra oligactis* and the regulation of spontaneous structural anomalies in the same species. A review of relevant literature and certain conclusions drawn from the literature are contained in the two articles and they will not be repeated here except for a few citations bearing directly upon the subjects of longitudinal fission and regulation.

Hyman (1928) states (p. 73) "that fission in hydra is not a normal method of asexual reproduction but a mode of regulation of previously existing abnormalities." The writer (Turner, 1952) observed (p. 107) that "the bifurcated condition of the apex which initiates longitudinal fission originates most commonly, and possibly exclusively, in buds which are in the latter stages of development and are still attached to the parent." Chang, Hsieh and Liu (1952) found that fission occurred in buds in a ratio of about one in a thousand. The writer has found since 1952 in large mass cultures and also in isolated strains that twinning in buds is fairly common and that true twinning originates only in buds. Conditions resembling twinning have been discovered in mass cultures but when these specimens are followed through day by day some of them have proved to be cases of actual twinning but others have proved to be specimens undergoing regulation. The complexes are often resolved by fusion of the members, absorption of one member by the other or one member forms a new foot and separate from the other.

The term "twinning" is used here advisedly and has most of the features common to monozygotic twinning in higher forms. In both hydra and higher forms an embryonic mass, produced asexually as a bud in hydra, divides and two individuals proceed separately to develop into whole equal animals. The separation of the two individuals may be incomplete in higher forms and produce monstrosities in various degrees. In hydra, individuals will become separated eventually because they have retained to a remarkable degree the capacity for regulation during which multiple complexes are reduced to simple individuals with the characteristics of the parent animal. In hydra the initiation of twinning may occur at any stage of bud development but it is not completed by the time of detachment from the parent. Another process comparable to twinning in its end result is double budding. In this process two buds in precisely the same stage of development arise at the same time in the budding zone of the parent as separate masses, usually on opposite sides of the body stalk, and after developing at the same rate they become detached at the same time. At no stage are they connected and they differ from the ordinary bud in that ordinary buds arise in a sequence, differ from each other in stages of development and become detached from the parent at different times.

MATERIALS AND METHODS

Specimens were separated from a large mass culture and were placed individually in bottles of about 100 cc. capacity. They were fed with Entomostraca daily and the water in which each lived was replaced daily with water from the large tank in which the mass culture was maintained. Daily records were kept of each specimen concerning its behavior, its reproduction and its periods of physiological depression.

In order to secure an accurate determination of the extent of twinning in the members of the mass culture, single non-twinning specimens were separated from the mass culture and a record was kept of all of the young produced by budding. The record of each was followed for 20 to 30 days and the proportion of twinning to non-twinning buds was determined from the totals. The process was repeated three times at two-month intervals.

When twinning buds were discovered they were isolated after they had become detached from the parents and daily records were kept of buds produced by the single undivided portions and by the bifurcated portions of the twin. The total number of buds produced by the complex was secured in this way and a comparison could be made between twinning individuals and single individuals of the rate of bud production. The proportion of single to twinning buds was obtained and could be compared to the production of single and twinning buds in control specimens which had no previous history of twinning. The possibility that certain strains had a tendency to produce a high proportion of twinning buds could be examined from the results.

PROPORTION OF TWINNING TO NON-TWINNING INDIVIDUALS

Any estimate of the proportion of twinning to non-twinning individuals based upon bifurcated specimens taken from a large mass culture would be invalid because a part of the individuals in the bifurcated state would not be cases of genuine twinning. Some of them would be mature individuals in which a bifurcation had arisen at the apex during or immediately after a period of depression. By the selection of healthy single individuals and the recording of each bud which arose in these individuals, it was possible to know whether any suspected case of twinning was real and, if it was real, to know also the place of its origin and its subsequent history. Twenty-five single individuals selected from the large mass culture were given the most favorable conditions for reproduction and within a period of 20 to 30 days they produced a total of 705 buds of which 14 were twins. The twinning buds represented 1.98 +% of the total. Two months later 11 single individuals, selected from the same mass culture and maintained in the same way, produced 274 buds of which 4 (or 1.45 +%) were twins. The operation was repeated two months later and 12 specimens produced 299 buds of which 6 (or 2.01%) were twins. In the three samples, 48 isolated individuals produced 1278 buds of which 24 or 1.87 +% were twin buds.

Twenty of the twin buds were isolated when they were detached from the parent and a record was kept of the production of buds by each specimen for 13 to 22 days. A total of 523 buds was produced of which 19 (3.63 +%) were twin buds. During this time ten of the twinning specimens produced only single buds but one of them produced three twinning buds (Fig. 3) and several produced two twinning buds

(Figs. 1 and 2). Since the reproducing individuals derived from twinning buds produced nearly twice as many twinning buds as individuals taken at random from the mass culture, it may be inferred that an inherited tendency for bud twinning existed in the strains showing unusual bud twinning.

Origin and Structure of Twins

Twinning has been observed in buds at various stages of development. In the earliest cases the bud develops a bilobed condition almost from the moment of its origin as a bud (Fig. 17). Each lobe then elongates and differentiates (Figs. 4 and 5) and by the time of detachment from the parent the two members of the twin are separate except at the base (Fig. 6). If the specimen indicated in Figure 6 were to be found in a mass culture without its previous history being known, it might be interpreted as a case of apico-basal fission but it is actually one in which no fission is involved up to the time of its detachment. Rather, two separate portions of the original single bud have undergone parallel development. After detachment the twinning individual undergoes fission and the two members are separated. It may be stated that twinning gives rise to an anomalous state which is resolved by regulation (fission).

A bud may develop as a single unit for some time and then give rise to dual masses at the apex (Fig. 7). The basal single portion elongates somewhat and at the same time the members of the divided portion will elongate and differentiate. After detachment from the parent (Fig. 8) fission of the remaining common stalk and the base occurs. Regulation (fission) is involved to a greater extent than it is in cases of very early bud-twinning.

Twinning occurs at a late stage of bud development in some cases (Fig. 9) and when the bud is detached from the parent only the apical portion of the bud is bifurcated. The regulation process (fission) occupies a longer part of the entire period during which two complete individuals are formed from a single bud.

A bud occasionally gives rise to three instead of two units. In the case illustrated in Figure 11 three hypostomes, each with a circle of tentacles, arose in a late stage of bud development. Before the bud was detached the hypostome of one unit was absorbed by another but the tentacles of the absorbed unit remained intact. When the bud was detached it resembled the one shown in Figure 10 except that one of the terminal units possessed supernumerary tentacles. In this case three processes of regulation would be involved before two normal single individuals were formed from the complex bud. Absorption of the hypostome occurs before bud detachment. Apico-basal fission would separate the two members after detachment of the complex from the parent, and still later fusion of tentacles would occur in the individual having supernumerary tentacles until the normal number of tentacles was produced.

Secondary twinning, *i.e.*, twinning in one or both members of a specimen which is itself a twin, has been observed a number of times. In Figure 12 a single speciment is illustrated together with a twinning bud. The primary twinning occurred when the bud was half developed and the two members of the twin bud proceeded to elongate and differentiate. At a late stage in differentiation one of the members divided at the apex. When the bud was detached it consisted of a common stalk and foot and a stalk divided in its terminal half. Also one member of the di-

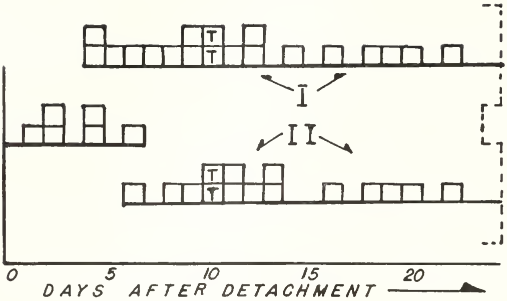


FIGURE 1

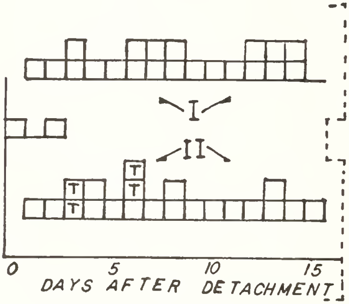


FIGURE 2

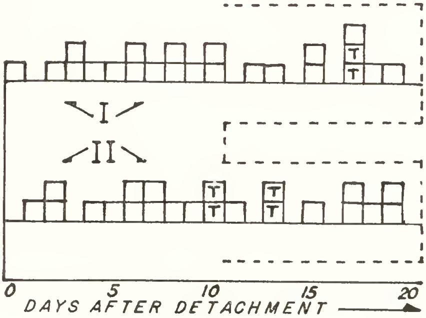


FIGURE 3

FIGURE 1. Reproduction by budding in twinning specimen shown in Figure 10. Period covers time from detachment from parent to time of separation of twins by apico-basal fission. Fission was completed in 24 days. Squares indicate new buds per day. Reproductive record of single portion shown at left, of divided portions, in I and II. T indicates twinning in a bud.

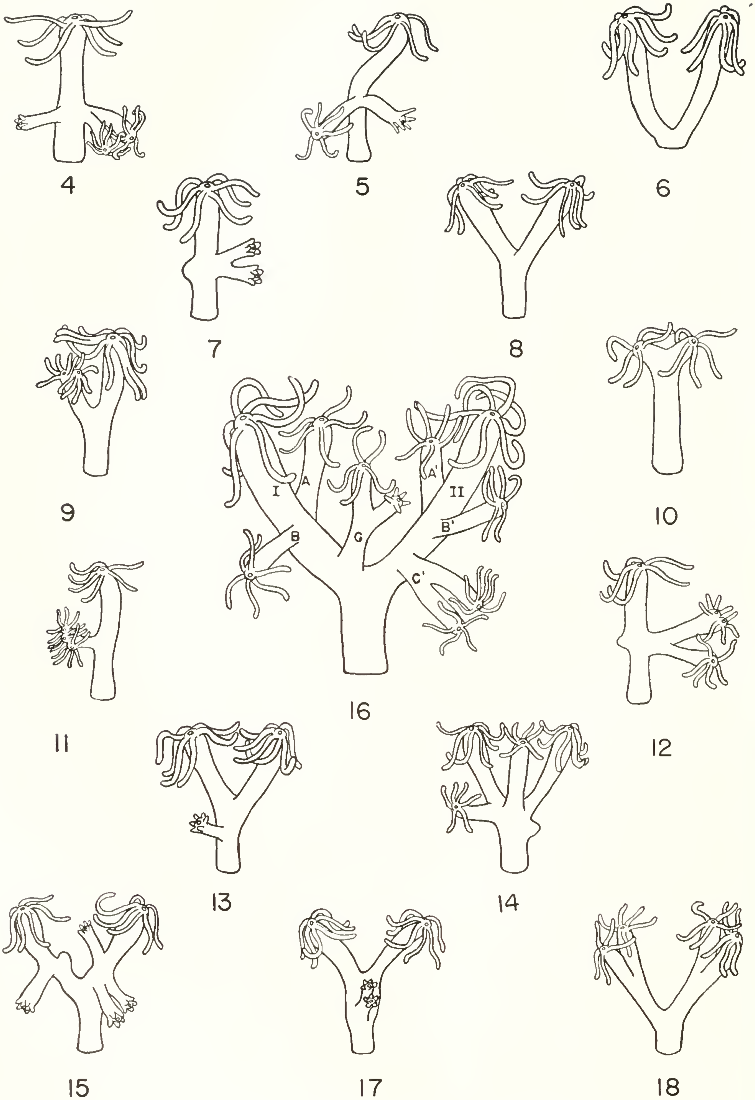
vided portion had two hypostomes. In the member which was divided at the end, fusion occurred and within several days a single hypostome was present, surrounded by eight tentacles. Fission separated the two primary members eighteen days after detachment of the twinned bud from the parent. Figures 15, 16 and 17 illustrate cases in which twinning buds had become detached from the parent and were actively reproducing. Fission which will separate the primary twins is in progress. A new bud appearing on one of the stalks of the specimen (Fig. 17) is twinning in an early stage producing a case of secondary twinning. Similar situations are illustrated in Figures 15 and 16 except that in both cases fission has divided the detached primary twin down through the budding zone and the secondary twinning buds are arising from divided instead of the common undivided parts of the stalk. A remarkable case is illustrated in Figure 18. The complex of four members arose as a single bud which twinned at an early stage. After a short period of elongation of the two members each member twinned again. Elongation of each of the four members occurred and each differentiated with a full complement of tentacles and a hypostome. After the complex was detached from the parent the primary division was completed by fission and some days later a secondary fission separated the members of the secondary twins. No fusion of any kind occurred. It appears in comparing the cases shown in Figures 9, 10 and 18 that secondary twinning is likely to be effective and to produce separate individuals if it occurs early and the members are able to grow and to differentiate a full complement of normal parts, but if the twinning occurs very late one member is likely to be absorbed by the other.

Complexes are encountered occasionally which involve at the same time multiple twinning and other unusual features. They present a complicated appearance and the units can be accounted for only if a complete record has been kept from the time of origin of the individual producing the complex. A case in point is the complex illustrated in Figure 16, and the history of this case is as follows. A bud arose from a normal single hydra and proceeded to twin when half developed. The twinning bud became detached from the parent and began to produce buds of its own in the common stalk almost immediately. Fission of the twinned individual was carried basally and passed through the budding zone. At the moment illustrated in Figure 16, I and II represent the divided portions of the individual described. Portion I has three buds, A, B and C. Bud C is going through the unusual process of giving rise to a new bud before it has become detached from the parent. Portion II is giving rise to buds A′, B′ and C′. Bud C′ twinned at an early stage and has not become detached. The subsequent history of the complex involved further reproduction and a resolution into single normal units. Portions I and II gave rise to new buds while they were being separated by fission and thereafter appeared as normal single budding individuals. Bud C became detached within a few hours and became a single reproducing individual. Twinning bud C′

The notch in the broken line at the right indicates the time of separation by fission of the members of the twin.

FIGURE 2. Reproduction by budding in twinning specimen divided for half of its length when detached from parent as in Figure 8. Fission complete on 16th day. Symbols as in Figure 1.

FIGURE 3. Reproductive record in twinning specimen divided for ⅞ of its length at time of detachment from parent. Reproductive period extends from time of detachment of twinned bud from parent (0 days) to completion of fission (10 days) and in addition, 10 days after completion of fission. Symbols as in Figure 1.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Figures 4–18.

became detached in several hours and nine days later fission had separated the members of the twin. In the meantime twinning bud C′ was budding off new individuals. A total of eighteen days were required from the time of the appearance of the original bud for the formation of the complex and for its resolution into single individuals.

Reproduction in Twins

Reproduction in twins was studied for the purpose of determining whether the process of twinning and subsequent regulation interfered with, or in any way affected, the process of reproduction and also to compare the over-all production of new individuals in twins with that of single individuals. Three cases were selected for illustration on the basis of the degree of apico-basal division in the twins at the time of the detachment from the parent.

In case 1 (Fig. 1) the twinning bud was detached from the parent when the apical part was divided for approximately one-third of the entire length of the bud. The budding zone in the common stalk was not divided at the time of detachment and three individual buds were produced within the first four days on the common stalk. Buds produced by the common stalk are shown by blocks in the middle line of the three lines of blocks. On the fourth day after detachment, fission

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Figure 4. Single parent with bud which twinned at an early stage and developed as two individuals separated except for basal ⅛ at time of detachment.

Figure 5. Single parent with bud which twinned at an early stage and was almost completely divided at time of detachment from parent.

Figure 6. Bud shown in Figure 4 after detachment from parent.

Figure 7. Young bud which twinned when half developed.

Figure 8. Bud shown in Figure 7 after detachment from parent.

Figure 9. Bud which twinned at a late stage of development. Supernumerary tentacles and fusing pairs of tentacles in parent indicate that parent is an incompletely regulated specimen in which two apical units have fused. Bud was divided for ⅛ of its length when detached.

Figure 10. Bud shown in Figure 9 after detachment from parent. Apico-basal fission was completed in 24 days. See Figure 1.

Figure 11. Multiple division of the apical end of a bud. After detachment one of the hypostomes fused with the nearest hypostome. The two remaining units were separated by apico-basal fission.

Figure 12. Twinning occurred at an intermediate stage of bud development and secondary twinning occurred late in the development of one of the units. The secondary twinning was reduced by absorption of one member by the other.

Figure 13. Budding in the undivided portion of a specimen which twinned as a bud and is undergoing apico-basal fission. See Figure 2.

Figure 14. Same specimen as in Figure 13. Apico-basal fission has proceeded down to the budding zone.

Figure 15. Same specimen as in Figure 13. Apico-basal fission has proceeded through the budding zone down to the stalk. One of the new buds is a twin.

Figure 16. Specimen 8 days after it was detached from parent as a twinned bud divided for about ½ of its length. Apico-basal fission has proceeded downward through the budding zone. Member I has three buds, A, B and C. C, not yet detached, is producing a bud. Member II has produced three buds, A′, B′ and C′. C′ is a twinning bud. Specimen produced 43 buds before fission was completed in 21 days.

Figure 17. Specimen which arose as a twinned bud and is now producing a twinning bud. Divided members of the new bud have unusual arrangement along apico-basal axis of parent.

Figure 18. Complete double twin. Specimen twinned in an early bud stage and each member twinned at a later stage before the four-member complex was detached from the parent.

had carried down to the budding zone and two new buds appeared on the common stalk and two upon one of the divided portions above the point of function with the other member. On the fifth day after detachment another bud appeared on the same member. On the sixth day one new bud appeared upon the common stalk and one each on the divided members. Thereafter no new buds appeared upon the common stalk and it is apparent that fission had carried down through the budding zone and that the time for its passage through the budding zone was three to four days. The time of the complete separation of the members of the twin was 23 days after the twinned bud had become detached from the parent. The time of separation is indicated by the notch in the broken line at the right of the figure. During the process of fission, during which the members were separated from each other, reproduction continued in each member at approximately the same rate as it would have occurred in a single individual.

Reproduction in a bud which had twinned somewhat earlier than the one shown in Figure 1 is represented in Figure 2. When the bud was detached from the parent it was divided apically for about one-half of its length, presenting the appearance of the specimen shown in Figure 8. The division point was within the budding zone and each member began to form new buds the day after the twin was detached from the parent. Fission carried down through the budding zone within three days during which time the common stalk produced two buds. The members of the twin were separated from each other 16 days after the twinned bud was detached from the parent and each produced buds continually during the regulatory process of fission.

A twin-bud specimen which was divided for about seven-eighths of its length when detached from the parent reproduced as shown in Figure 3. The specimen (Fig. 6) had twinned at an early stage and separate and complete budding zones were represented in each member. Each member began to give off new buds as soon as the specimen was detached from the parent and continued to do so for the ten days required for fission to separate the members. The reproductive history of each member for ten days after separation is shown at the right in the diagram. It will be noted that the rate of bud production in each member was about equal and that the rate is the same whether the members were attached to each other or separated.

It is apparent from the results that the process of twinning, and of fission which separates the members of a twinned individual after its detachment from the parent, do not affect the process of reproduction by budding. It may be added that there is no interference with the formation and maturing of spermaries. Buds, whether single or twinning, become detached from the parent within 48 hours after their first appearance if the parent is not in a state of depression, and spermaries are not formed until later. However, spermaries have been observed in both members of twinned specimens during the process of fission which later separates the members of the twin.

Duration of Fission

Fission, as the term is used here, refers to the process moving in the apico-basal axis by which the members of a twinned individual are separated. It occurs after the bud has been detached from the parent. The rate at which fission proceeds is quite variable and the duration of the process depends upon the degree of initial separation of the members at the time of detachment from the parent and upon the rate at which it proceeds. Four individuals divided at the apical end for a distance of one-fifth of the total length required, respectively, 17 days, 27 days, 38 days and 51 days for complete separation. The process moved through the apical third rapidly, and proceeded through the budding zone in three to four days. Fission moved at a slower rate through the body stalk at the basal end and lagged greatly in the region of the foot. In an extreme case 20 days were required for separation of the foot after fission had carried down to that point. Two specimens, each separated for one-half of the total length at detachment, required 23 days for the completion of fission. Three specimens divided almost to the base at the time of detachment required, respectively, 8, 9 and 11 days for complete separation.

Summary

1. In the specimens observed in pedigreed cultures, genuine twins arose only in buds.

2. Fission, regarded as a separate regulatory process in which twinning complexes are resolved into single individuals, occurs after the twinning buds have become detached from the parent.

3. In pedigreed cultures specimens arising as single buds produced 1278 buds of which 24 were twin buds. Specimens arising as twinning buds produced 523 buds of which 19 were twin buds.

4. Twinning may occur in a bud at any stage of development. An early twinning bud is deeply divided at the time of detachment from the parent and a late twinning bud is divided only at the apex.

5. Multiple twinning occurs occasionally in which one or both members of a twin bud undergo secondary twinning before detachment of the complex from the parent.

6. Bud production by a specimen arising as a twin bud is equal to that of a single individual as long as the budding zone is undivided. Bud production is doubled as the budding zone is divided by fission.

7. Completion of fission of a twin bud requires usually from 8 to 27 days but may take as long as 51 days in a depressed specimen. Fission proceeds rapidly at the apical end, passes through the budding zone in three or four days and is retarded most at the basal end of the body stalk and the foot.

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PROPERTIES OF THE CONNECTIVE TISSUE SHEATH OF THE COCKROACH ABDOMINAL NERVE CORD[1, 2]

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Hoyle (1952, 1953) has drawn attention to the continuous sheath which surrounds nerve fibers and ganglia of *Locusta* and other insects. He has described the structure of this sheath and demonstrated that its effectiveness as a diffusion barrier enables the nerves of *Locusta* to function normally despite wide variations in the ionic composition of the surrounding fluid. His valuable work indicates basic similarity in the membrane properties of insect nerve and nerve of vertebrates and of invertebrates other than insects. In the present study of the ventral nerve cord of the roach, Hoyle's conclusions are confirmed. Normally sheathed and desheathed cords were compared with respect to interference with nervous function by variation in total salt concentration, sodium deficiency, excess potassium ions and acetylcholine. Certain structural details were studied histologically.

Materials and Techniques

Adult male specimens of *Periplaneta americana* have minimal fatty deposits about the cord and were therefore used. For observations of the effects of ions on axonic conduction in the ventral cord, the head was crushed, and the cockroach was pinned, ventral side up, on a cork platform, with the legs taped down. Test solutions were applied and conduction examined in a segment of nerve cord comprising the fourth abdominal ganglion and the connectives between the fourth and fifth ganglia. Cuticle was removed over this region and a thin paraffin sheet was placed beneath the test segment (Fig. 1a). Drainage arranged from below the paraffin minimized mixture of hemolymph with the test solutions, which were perfused over the segment lying on the paraffin. In some experiments, it was possible to avoid cutting any large nerves or tracheal branches by locating the paraffin entirely under the connectives. Silver-silver chloride hook electrodes (Roeder, 1946) were placed below the cord and moved over the test area so that localized axonic block could be detected by changes in form of the compound action potential in the giant fibers (Fig. 1b).

Action potentials conducted into or through the test area could be elicited either directly via stimuli from a pair of silver electrodes (Fig. 1a, S_1) inserted under the nerve cord through a small cuticular opening near the first abdominal ganglion, or transynaptically via stimuli from a similar pair (S_2) inserted into the base of a cercus. An uninterrupted sequence of square pulses (0.5 per second; 0.2 msec.

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[2] The authors wish to acknowledge the able assistance of Miss Janice Green and Mr. Edward L. Raymond in the histological work.

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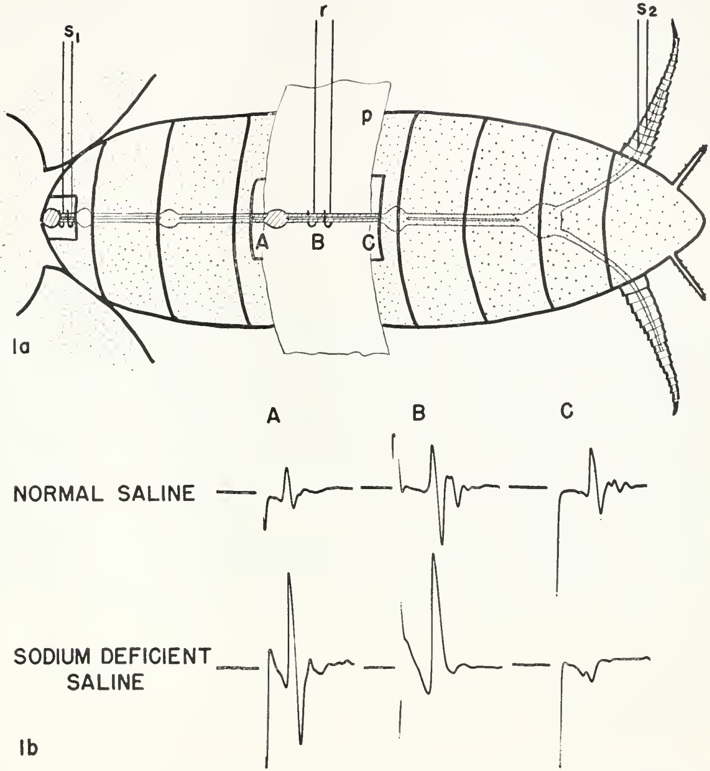

FIGURE 1. (a) S_1, stimulating electrode pair on cord near first abdominal ganglion; S_2, stimulating electrode pair on cercal nerve; p, paraffin sheet; r, recording electrode pair; A, B, C, sites in test area at which action potentials were recorded. (b) Records of action potentials at sites A, B, and C; stimulation at S_1 in a preparation desheathed at B. In sodium-deficient saline, the spikes are larger, due to decreased shunting by electrolyte. Localized block in the desheathed region between electrodes is indicated by the monophasic spike at B.

duration) was applied through S_1 except for brief periods when ascending conduction was checked through stimulation at S_2. In normal or potassium-free saline, this preparation responded uniformly well for many hours. "Normal" saline refers to Hoyle's (1953) basic mixture for *Locusta*, which proved most satisfactory in our experiments.[4] High potassium and potassium-free salines were made up as detailed by Hoyle.

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[4]

KCl	10 mM./L.	$MgCl_2$	2 mM./L.
NaCl	130 mM./L.	NaH_2PO_4	6 mM./L.
$CaCl_2$	2 mM./L.	$NaHCO_3$	4 mM./L.

The last abdominal ganglion, exposed as described by Roeder, Kennedy and Samson (1947), was the test object in acetylcholine studies. Electrical stimuli were applied to the cercal nerve at low frequency (0.5/sec.) and the postsynaptic response was led off near the fifth ganglion. The last ganglion was continuously perfused with saline, to which acetylcholine was added for tests.

The recording system consisted of a Grass P-4 preamplifier and a Dumont 304-A oscilloscope. Responses were photographed with a Dumont oscillograph record camera, type 297. Square pulses were delivered from a Grass S-2 stimulator.

Methods were developed for desheathing ganglia and connectives in the above preparations. These operations were most conveniently performed under a dissecting binocular at a magnification of 80 ×, using two pairs of fine-ground watchmaker's forceps. Lowering the saline level briefly caused a barely perceptible crinkling of the sheath about the ganglion. The sheath was lifted, torn, and gently pulled away from the entire dorsal surface of the ganglion. The saline level was rapidly adjusted to prevent drying of the desheathed ganglion. This procedure in no way altered the character of the postsynaptic response (see Fig. 2). Desheathing the connectives was more difficult since conduction failed if even a brief drying occurred, and visualization of the sheath under saline took some practice. Stretching the desheathed connective had to be avoided. The sheath, once torn, could be rolled back along the connective, which it enveloped in stocking-like fashion. The sheath is quite elastic and constriction of the cord by the rolled-up sheath had to be avoided by proper tearing. The stripping, successfully accomplished, did not alter the nerve action potential. In normal saline, desheathed preparations responded without change for hours.

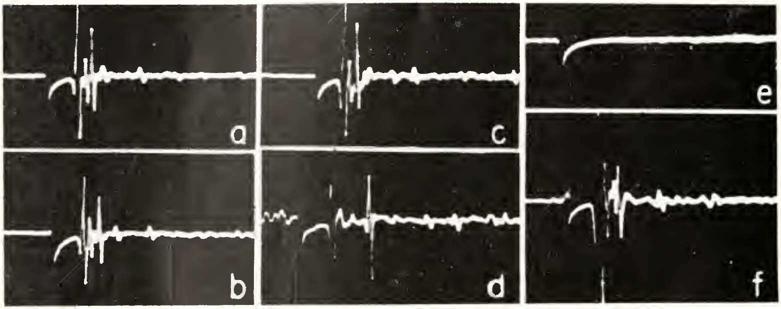

FIGURE 2. Cercal nerve stimulated. Postsynaptic responses recorded from abdominal cord near fifth ganglion. (a) In normal saline; last abdominal ganglion normally sheathed. (b) After 10 minutes in acetylcholine 10^{-2} *M;* normally sheathed. (c) After one minute in acetylcholine 10^{-2} *M;* desheathed. (d) After three minutes in acetylcholine 10^{-2} *M;* desheathed. (e) After four minutes in acetylcholine 10^{-2} *M;* desheathed. (f) After washing in normal saline; desheathed.

The posterior portion of the abdominal cord, containing the fifth and last abdominal ganglia, was examined in 6-micron serial sections. A mercuric chloride-acetic acid mixture provided most satisfactory fixation. Ester wax (Steedman, 1947), with increased paraffin content, proved an excellent embedding medium. Conventional staining techniques included: Mallory's triple stain, Masson's trichrome, Gomori's chromium-hematoxylin-phloxin (Gomori, 1941) and Holme's silver as modified by Batham and Pantin (1951). Desheathed specimens were fixed after physiological tests.

A series of cords was stained by Laidlaw's method as described by Krnjević (1954) after the fifth ganglion and one half of the connective between fifth and sixth ganglia had been desheathed. The living cord was placed in 0.5% silver nitrate and observed under strong illumination. One of this series was fixed, sectioned and counterstained with Mallory's (Fig. 3b).

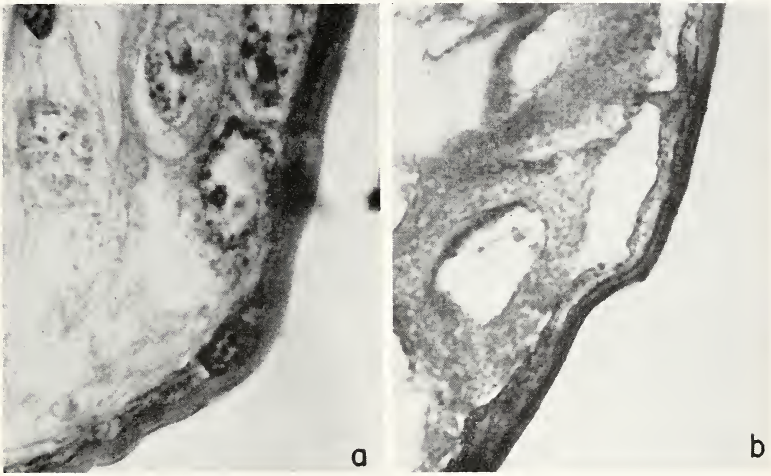

FIGURE 3. (a) Normally sheathed last abdominal ganglion stained with Masson's trichrome. Note outer homogeneous layer and inner nucleated granular layer of sheath. (b) Normally sheathed last abdominal ganglion after silver nitrate treatment. Note adherence of inner and and outer sheath layers in region of shrinkage. Note silver granules in sheath, absence of granules in interior of ganglion. Counterstained with Mallory's triple stain.

OBSERVATIONS

Osmotic changes

The intact, normally sheathed cord was not observed to swell or shrink and nervous conduction was unaffected when total solute concentration was reduced to the equivalent of 0.140 M NaCl by dilution or increased to 0.180 M by adding NaCl or sucrose to normal Hoyle's saline (0.156 M). Desheathing in the hypotonic (0.140 M) saline resulted in gross swelling and functional impairment. Synaptic conduction failed totally. Desheathing in the saline hypertonic to Hoyle's saline (0.180 M) resulted in no immediate structural or functional alternation.

High potassium saline

In Table I are listed typical times for total conduction block on perfusing a previously untreated cord with 0.180 M KCl or Hoyle's balanced saline containing 140 mM/L. K^+. Recovery was followed in K^+- free saline.

Blocking was more rapid in 0.180 M KCl. This could well be attributed to the absence of sodium and other ions rather than higher K^+ concentration. Desheathing obviously reduced recovery time as well as blocking time. The presence of the cut ends of small nerves (severed in dissecting the fourth ganglion) did not alter the blocking time appreciably.

TABLE I

Effect of potassium ions on impulse conduction in intact and desheathed cords

A. Intact segment

Solution	Blocking time	Recovery time	Type preparation
0.180 M K^+	12 min.	10 min.	Connective only
0.180 M K^+	15 min.	10 min.	Ganglion included
0.180 M K^+	18 min.	20 min.	Ganglion included
140 mM./L. K^+	30 min.	25 min.	Ganglion included
140 mM./L. K^+	22 min.	33 min.	Ganglion included

B. Test segment desheathed at B (see Fig. 1a)

Solution	Blocking time	Recovery time	Type preparation
0.180 M K^+	10 secs.	4 min.	Ganglion included
140 mM./L. K^+	60 secs.	2 min.	Ganglion included
140 mM./L. K^+	90 secs.	2 min.	Ganglion included

Repeated applications of high K^+ solutions to intact cord segments led to increasingly rapid block and delay in recovery. This effect was not seen in desheathed nerve.

Sodium-deficient saline

Substitution of sucrose for the sodium of the normal saline did not affect the normally sheathed segment in experiments continued several hours, although this caused conduction block in a stripped preparation within 30 seconds (Fig. 1b). Recovery was complete within two minutes in normal saline.

Acetylcholine

Treatment of the intact ganglion with extremely high concentrations of acetylcholine (10^{-2} M) did not alter synaptic responses (Fig. 2 a, b), as had been reported by Roeder (1948). However, effects of 10^{-2} M acetylcholine on synaptic function in the desheathed ganglion were usually rapid and pronounced. In two of a series of seventeen experiments, only a moderate decrease in synaptic response was noted. In all others, within one to five minutes bursts of asynchronous action potentials were followed by synaptic depression and block. Figure 2 shows the rapid and easily reversible block of a ganglion desheathed in 10^{-2} *M* acetylcholine. The lowest effective concentration of acetylcholine was between 3 and 5×10^{-3} *M* in the uneserinized desheathed ganglion. After eserine, acetylcholine between 10^{-4} *M* and 10^{-3} *M* blocked the synapse. No effect on axonic conduction was noted.

Sheath structure

Since the foregoing experiments demonstrated the functional importance of the sheath, an attempt was made to examine its mechanical properties and structure.

It was extremely difficult to penetrate the sheath of the intact nerve cord with any object. Even a finely tapered capillary microelectrode merely dimpled the surface and broke. After desheathing, penetration of the cord and individual neurons presented no difficulty. During dissection, the sheath felt tough and elastic. As has been mentioned, in some dissections the sheath severely constricted the cord. The almost explosive bulging-out of nerve substance through a small hole made in the sheath when the preparation was immersed in hypotonic saline strikingly illustrated the mechanical resistance the sheath offers to swelling. Injected air bubbles were trapped beneath it. Histologically, the sheath was continuous and clearly double-layered. The outer, almost homogeneous layer, two to five micra in thickness, stained deeply with aniline blue. Occasional nuclei were evident in this layer, possibly representing fibroblasts. The inner layer consisted of granular squamous epithelial cells, one to three micra thick. The flattened nuclei of the inner layer were prominent in both cross and coronal sections (Fig. 3a). (The outer layer referred to above corresponds to the homogeneous, non-cellular, neural lamella described by Scharrer (1939) and Hoyle (1952), the inner layer to the thin, continuous cytoplasmic cylinder which Scharrer terms the perineurium and Hoyle terms the perilemma.) These layers were closely adherent to one another and pulled away together from the nerve substance when the preparation was desheathed or when shrinkage occurred in fixation (Fig. 3b). No other continuous structures lay external to these layers in our intact preparations. These two layers were always absent in areas functionally desheathed.

It should be mentioned that within the nerve cord itself, fibrous investments, almost capsular in appearance in silver-stained sections, but probably discontinuous, surrounded certain large cell bodies and groups of cell bodies. These fibrous investments may correspond to the glial elements described by Scharrer (1939). The giant axons were individually encased in a delicate (one micron in thickness) sheath-like structure throughout their length.

When a freshly-dissected, unfixed cord was observed during soaking in silver nitrate, the normally sheathed areas showed at first a fine network of black lines, then became stippled and blotched in appearance and finally almost uniformly black. The blackening was close to the surface and the deep black layer could be lifted off by desheathing. Desheathed cord soaked in silver nitrate did not blacken superficially, but individual neurons could be observed to stain black in these preparations. Sections of the intact last abdominal ganglion, treated while fresh with silver nitrate, showed black granules concentrated in the surface layer corresponding to the sheath (Fig. 3b), while the desheathed fifth ganglion, after similar treatment, showed granules distributed throughout.

Discussion and Conclusions

The sheath surrounding the nerve cord of *Periplaneta* clearly limits diffusion of ions, as Hoyle (1953) showed in *Locusta*. However, in efficiency of sheath function as measured by total time required for block in excess potassium, the intact *Periplaneta* cord is more comparable to the amphibian sciatic nerve, which will block in 13–20 minutes in isotonic KCl (Lorente de Nó, 1947; Feng and Liu, 1949) than to *Locusta* nerve, which resists block as long as four hours in saline containing 140 mM potassium, provided the tracheal supply is undamaged. Hoyle pointed out the importance of adequate oxygenation in maintaining sheath function. It may be that in our preparation, since abdominal movements were severely restricted, oxygenation through the tracheal supply was insufficient to maintain sheath function although efforts were made to avoid all damage to the tracheal supply.

The retardation of potassium diffusion is a two-way function of the sheath, judging by the large decrease in recovery time as well as blocking time in desheathed cord. The cumulative effect of repeated high potassium solutions, as well as the appearance of the silver nitrate-treated cord (Fig. 3b), suggests that ions penetrate the sheath readily but are accumulated there. Shanes (1954) showed with tracer techniques that the sheath of the frog sciatic nerve is entirely responsible for retardation of ion diffusion outward as well as inward.

Desheathed *Periplaneta* cord is soon blocked by sodium deficiency, although conduction in the intact cord is unaffected for long periods in sodium-free saline. This, then, is direct evidence that insect neurons are similar to the neurons of other groups not only in potassium sensitivity but in their susceptibility to inactivation by sodium deficiency, and that it is the sheath which masks this susceptibility just as does the sheath which invests vertebrate nerve (Huxley and Stämpfli, 1951).

Although much pharmacological evidence suggests the cholinergic nature of the synapse between cercal nerves and giant fibers in the last abdominal ganglion of *Periplaneta*, not even very high acetylcholine concentrations affect the intact ganglion. This total lack of effect is clearly due to restriction of acetylcholine penetration by the sheath. However, effective concentrations of acetylcholine are high even in desheathed preparations so that the synaptic specificity of the acetylcholine action is still in question. (See Twarog and Roeder, 1957 for further data.) The sheath-like structure which invests the giant fibers may represent another barrier to ion diffusion, or the synaptic terminations of the cercal nerves may be otherwise "protected."

Water diffuses through the sheath quite readily, as is evident from the immediate swelling-out of nerve substance which occurs when a small portion of the sheath is removed while the cord is soaking in hypotonic saline. It is obvious that the physical restraint exerted by the sheath in preventing swelling limits total water uptake and preserves structural integrity. Lorente de Nó (1952), Shanes (1953) and Krnjević (1954) have emphasized the possibility that the mechanical rigidity of the vertebrate sheath may serve an osmoregulatory function. The fluctuations in hemolymph water in insects are often very great (Mellanby, 1939), and an osmoregulatory function of the sheath may be as important as its role in salt regulation.

The actual site of ion regulation by the sheath is in one or both of the two layers described above, which were also described by Hoyle (1952) in *Locusta.* Huxley and Stämpfli (1951) suggested that ion regulation by the frog sheath is a function of the innermost epithelial layer first described by Ranvier in 1876 rather than the loose connective tissue of the epineurium. Krnjević (1954) showed conclusively that this is true. Within the epineurium he described the two-layered perineurium: an inner continuous layer of squamous epithelium, 4–6 micra thick, with an external layer of comparatively undifferentiated connective tissue. In some regions of the sciatic nerve additional cellular layers were seen, but these were not continuous over the entire nerve. He succeeded in showing that the silver granules are most concentrated in the epithelial layer. The analogy in structure between the insect sheath and the frog perineurium is rather striking. It is likely that it is the squamous epithelial layer which fulfills the important regulatory function in the insect. Krnjević (1954) has discussed the importance of epithelial layers in regulation of diffusion through capillary walls and through the connective tissue sheaths which surround nervous structures in vertebrates. The inner, epithelial layer of the sheath may well bear some physiological and structural resemblance to the so-called blood-brain barrier.

It must be mentioned here that the desheathing technique, in addition to having utility as a method of studying this interesting ion-regulating structure itself, presents advantages in investigating insect nervous function. It has been employed by Twarog and Roeder (1957) in pharmacological studies to avoid total failure of drug penetration or insufficiently rapid penetration to the site of action. Perhaps more important is the fact that it makes possible routine exploration of the insect nervous system with microelectrode techniques. Resting cell membrane potentials of 50 to 70 MV have been easily measured and sustained. Cell action potentials have been obtained but no systematic study has yet been made.

SUMMARY

1. A method is described for removing portions of the connective tissue sheath which invests the nervous system of the cockroach, making possible studies of sheath function and of basic physiological properties of the insect neuron.

2. This sheath restricts diffusion of potassium and sodium ions and of acetylcholine from the surrounding fluid to the interior of the cord.

3. The ability of the sheath to restrict swelling suggests a possible osmoregulatory function.

4. The functional sheath consists of an inner continuous squamous epithelial layer and an outer, almost homogeneous connective tissue layer.

5. This study confirms the conclusions of Hoyle (1952, 1953) with respect to ion regulation by the insect sheath, and indicates close functional and structural parallels in the sheaths investing insect and vertebrate nervous tissue as well as in the basic properties of the nervous tissues of insects and vertebrates.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY

1956

ABSTRACTS OF SEMINAR PAPERS

July 10, 1956

Amphibian yolk: chemistry and ultrastructure. Paul R. Gross.

The amphibian yolk platelet is a complex structure, both chemically and physically. Intact washed platelets contain protein, lipid, non-protein nitrogen, organic non-protein phosphorus, and pentose, probably in PNA. DNA is absent, as shown by a negative diphenylamine reaction. The protein of the platelets is made up of at least three macromolecular species, probably more. Sedimentation experiments at a variety of pH and ionic strength levels show that the main component, vitellin, accounts for more than 85% of the protein, while the remainder is in the form of two apparently polydisperse populations of particles. The vitellin sediments at about 12 S.

Further kinetic studies of the reaction in which the yolk platelets are lysed by low concentrations of Ca^{++} strengthen the hypothesis that this reaction is mediated by a Ca-activated enzyme system which resides within the platelet. As the concentration of Ca is lowered, the dissolution reaction undergoes a change of kinetic order from one to zero. Such a situation would not be expected were the solubilization a result of simple salting in, as is indeed the case in uniunivalent salts at ionic strengths greater than 0.4. The Ca-dissolution reaction also has an optimum over-all ionic strength which lies far below the minimum value for dissolution by uniunivalent salts.

The platelets are solids in water, and can be broken with microneedles into insoluble fragments. As the ionic strength is raised, the platelets are seen to swell and assume a more isodiametric shape, and at this point, preceding complete disruption, they are seen to be surrounded by a membrane. We have been able to photograph this "ghost"-like structure with the electron microscope.

Amphibian yolk: the phosphoprotein phosphatase system. Sylvan Nass.

The "PPP'ase" activity of yolk platelets isolated from ovarian frog eggs was studied under a variety of experimental conditions. The pH optimum for platelets incubated in NaCl of ionic strength between 0.1 and 0.4 was 4.9. The temperature optimum for 90-minute incubations was 45° C. In systems to which $CaCl_2$ ($\mu = 0.25$) had been added, the pH optimum was 4.3, while the temperature optimum was at 37° C. Relative to the uniuvalent salt, $CaCl_2$ acts in these systems as an inhibitor of P release. The Ca^{++} solubilizes the platelets, but no correlation between this process and P release was found.

Differences in PPP'ase activity between platelets incubated in NaCl at $\mu = 0.1$ and $\mu = 0.4$ can be directly related to the structural integrity of the platelet. At low ionic strength, the platelets usually remain intact during the incubation periods at the chosen temperatures and pH. Increase in ionic strength causes rapid dissolution of the platelets. Variations in temperature, pH, and time of incubation also determine the solubility of these structures. In all systems in which solubilization occurs (no Ca present), there is an increase in P release over that in control systems with platelets intact.

Kinetic studies show that the P release from intact platelets at any time is approximately half of that from platelets solubilized at the outset of an experiment. If then the intact platelets are dissolved by any means other than adding Ca^{++}, a sharp rise in the ($P_{inorg.}$) is obtained, bringing this value to that in the system solubilized at zero time. This may be interpreted in terms of a membrane-like diffusion barrier investing the platelet which retards release into the bulk phase of the hydrolyzed phosphate.

The relation of gonad hormones to x-irradiation sensitivity in mice. JOAN WOLFF AND ROBERTS RUGH.

A single injection of estradiol benzoate was made I.P. 10 days before whole body x-irradiation exposure to an LD/50/30 day level of 49 normal intact male CF_1 mice and 41 mice which had been castrated by surgical excision. Fifty other castrates and fifty intact mice, all of the same age, received no injections. Lethality data were collected for a period of 30 days following the irradiation. When the percentage survival is plotted against days post-irradiation, it is found that the intact male controls, which received no hormone injection, all died by the 17th day. At 30 days post-irradiation some 38% of the castrated males were still alive, indicating an adverse effect of the presence of the male hormone, testosterone, in the controls. Maximum survival was found in the castrated males which had also been injected with estradiol benzoate. The normal intact males which had been injected with this hormone showed a survival value almost as good, namely 63.3%. Thus the injected female hormone must have counteracted almost entirely the adverse effects of the male hormone. The differential in survival of male and female mice, when exposed to whole body penetrating x-irradiation, seems to be due in part to the gonadal hormones present. Intact males, with the normal quota of testosterone, have less survival value than do castrate males. Further, castrate males injected with the female hormone, eat radiole benzoate, exhibit maximum survival, approaching that of the intact female.

GENERAL SCIENTIFIC MEETINGS

AUGUST 20–22, 1956

Abstracts in this section (including those of Lalor Fellowship Reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read By Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Experimental hypothermia and carbon dioxide production in the white rat. C. LLOYD CLAFF, FREDERICK N. SUDAK AND NAOMI R. STONE.

A technique was described whereby carbon dioxide production could be measured in conscious rats utilizing barium hydroxide, N-butyl alcohol and thymolphthaline indicator. The efficacy of this method was tested with a carbon dioxide generator. Carbon dioxide up to seven times the output of a normal rat was generated, and 100% of this amount was collected.

Twelve-hour fasted male white rats were injected with Thorazine; 2,4-Dichlorophenoxyacetic acid; and a combination of the two drugs. Studies at room temperature (24° C.) revealed that a combination of Thorazine and "2,4 D." decreased the rectal temperature of a white rat from 36.3° C. to 31.8° C. with a diminution in carbon dioxide production of 30% in five hours. This was in marked contrast to the temperature-carbon dioxide relationship in animals which received only Thorazine. The rectal temperatures of these rats were reduced 6.0° C. in seven hours with an increase of 33% in carbon dioxide output.

One and one half hours after injection, animals were subjected to 2.0° C. Twenty minutes later, CO_2 and temperature measurements were taken. Normal rats increased their CO_2 output 136%, and maintained normal body temperatures. Animals receiving "2,4 D." and Thorazine in combination showed no significant change from normal output in their carbon dioxide, and a decrease in rectal temperature of 6.5° C. Thorazine-injected rats increased their CO_2 output

91% and rectal temperature dropped 5.5° C. Animals injected with "2,4 D." could only increase CO_2 output to 54%. A drop of 3.0° C. in rectal temperature occurred. All animals survived, and normal behavior patterns were observed within 24 hours.

The possibility of inducing hypothermia by interfering with the temperature regulating mechanism of a homoeothermic animal by means of drugs was discussed.

Sperm entry in Hydroides hexagonus (Annelida) and Saccoglossus kowalevskii (Enteropneusta). ARTHUR L. COLWIN, LAURA HUNTER COLWIN AND DELBERT E. PHILPOTT.

The authors have demonstrated previously (1954, 1955, 1956) that in a number of species the acrosome filament of the spermatozoon penetrates into the egg proper. The present species were selected for electron microscope studies because their eggs are essentially jellyless and their spermatozoa make direct contact with the egg membranes. Moderate polyspermy was induced in order to increase the chances of finding examples of sperm-egg association in thin sections. It is not established whether or not the spermatozoa here described were the specific ones which initiated the activation of the eggs studied.

In Hydroides, electron microscope photographs show that at the earliest contact between spermatozoon and egg the acrosome touches the thin, hexagonally patterned, layer which surrounds the much thicker vitelline membrane. Subsequently this thin outer layer seemingly rises up around the sperm head, even before a major part of the sperm head has entered the membrane. The vitelline membrane, too, appears to rise up around the sperm head, thickening in this vicinity. Near the acrosomal region of spermatozoa which appear to have breached the vitelline membrane completely, the perivitelline space is almost twice as wide as elsewhere. The above described effects suggest some lytic action stemming from the spermatozoon.

In Saccoglossus, electron microscope photographs show that acrosome filaments penetrating the outer and vitelline membranes are spiral in shape. The outer egg membrane is pitted wherever spermatozoa adjoin it. These pits are interpreted as resulting from erosion of the membrane by a lysin from the spermatozoon, demonstrating this lytic action at the level of the individual spermatozoon. The authors previously reported (1954) such pits in relation to sperm entry in living material as seen by light microscopy.

Electron microscope studies of the egg surfaces and membranes of Hydroides hexagonus (Annelida) and Saccoglossus kowalevskii (Enteropneusta). LAURA HUNTER COLWIN, ARTHUR L. COLWIN AND D. E. PHILPOTT.

With light microscopy, the vitelline membrane of living Hydroides eggs appears thick and uniform. Early in fertilization this membrane changes little and fails to elevate rapidly. The perivitelline space does not enlarge rapidly. With electron microscopy, sections of unfertilized eggs show a wide felt-like vitelline membrane closely surrounded by a single layer of very evenly spaced minute bodies linked in hexagonal pattern by fine threads. Similar threads link the layer to the membrane. Larger, fewer, microvilli project regularly from the egg proper, deeply penetrating the vitelline membrane. In cross section, at least the broader proximal part of the microvillus shows a deeply staining periphery encircling a central unstained region. No well-defined *layer* of cortical granules is seen. No great change is observed following fertilization. However, a narrow perivitelline space does develop; across this the microvilli project into the membrane for some time.

Light microscopy shows two membranes surrounding the living unfertilized Saccoglossus egg. Sections reveal a well-defined cortical granular layer. At fertilization both membranes elevate rapidly. The inner one (fertilization membrane) successively thickens, apparently receives additional material from the cortical layer, and becomes compressed. Simultanously, the egg surface forms short-lived irregular protuberances, the cortical layer disintegrates, hyaline granules and threads erupt from the egg into the rapidly enlarging perivitelline space. In living eggs much of this material becomes invisible, though a border of hyaline granules persists close to the egg. Sections show a reticulum in the perivitelline space. Electron microscopy verifies the above observations, shows release of cortical granular material, and also reveals stout microvilli between the egg and the vitelline membrane but not penetrating the latter. Upon fertilization these microvilli elongate, apparently detaching from the membrane; their ultimate fate is unknown. Cross sections of elongated microvilli show a well-defined circular central structure apparently surrounded by eight dot-like bodies.

Ionic regulation in the fiddler crabs, Uca pugnax and U. pugilator. JAMES W. GREEN, MARY HARSCH, LLOYD BARR AND C. L. PROSSER.

Three species of *Uca* (*pugnax, minax* and *pugilator*) like *Pachygrapsus* but unlike *Carcinus* and *Callinectes* were found to have lower concentrations of Na in urine when adapted to 175% than to 100% S.W. In *U. pugnax* and *pugilator,* the two species selected for more detailed study, the total osmotic concentrations of both urine and serum were slightly higher in crabs from the 175% S.W. than those from the 100%. Analyses of urine and serum from both groups for Na, Mg, Ca, K, Cl and SO_4 showed a cation deficit in the urine of about 20% which was found to be largely NH_4. Although the serum Na values of both groups were approximately the same there was 30 to 40% less Na in the urine of crabs in the 175% S.W. and the cation deficit thus incurred was made up by the excretion of about 3 times as much Mg as was found in the urine of the 100% crabs. When both groups were placed in S.W. containing Na^{24} the isotope was found to enter at essentially the same rate in each group. Tissue analyses of Na^{24}-labeled crabs indicated that Na was not being stored. When Na^{24}-labeled crabs were washed in S.W. without isotope, placed in dry finger bowls for short periods and samples of gill fluids analyzed, it was found that crabs from the 175% S.W. contained a higher proportion of Na^{24} in their gill fluids than the 100% group. This was interpreted to mean that while both groups lost Na through the gills the crabs in 175% S.W. excreted more by this route, thus accounting for the lowered urine Na values found.

Electron microscopy of the mitotic apparatus in dividing Arbacia eggs. PAUL R. GROSS, DELBERT E. PHILPOTT AND SYLVAN NASS.

In the course of an investigation of the mechanisms of sol-gel transformations in dividing cells, it became a matter of interest to determine the nature of the material which constitutes the mitotic spindle. Considerable data concerning this problem have been reported from Mazia's laboratory, but good, high-resolution electron micrographs of the achromatic figure are lacking. We have devised a new method for observing this structure with the electron microscope. The basis of the procedure is the observation of Mazia and co-workers that dividing cells can be killed with cold 30% ethanol without too extensive denaturation of cytoplasmic proteins and without dissolution of the mitotic figure.

The cells are fixed in the desired stage of mitosis with 30% ethanol at − 10° C. for about 30 minutes, then transferred to 30% ethanol containing 1% osmium tetroxide at − 10° C. After fifteen minutes in the cold osmic-ethanol, the suspension is removed to room temperature and permitted to warm slowly. After about fifteen minutes more, the eggs are quickly sedimented down and washed with 50% alcohol, thence run through a series of alcohols for dehydration. The cells are embedded as usual in a mixture of methyl and n-butyl methacrylates, and sections are cut on a microtome designed for ultra-thin sectioning.

Using this procedure, we have been able to observe in detail the structure *in situ* of the metaphase spindle and of the chromosomes at the metaphase plate. The spindle appears to be a gel composed of elementary particles of widely varying diameter, with a mean value near 400 Å. At high magnifications, these particles are seen to be themselves composites of somewhat asymmetric particles of diameter ca. 30–50 Å.

Participation by actin in actomyosin contraction. TERU HAYASHI, RAJA ROSENBLUTH, PETER SATIR AND MICHAEL VOZICK.

Any mechanism of muscular contraction at the molecular level must take into consideration the behavior of the muscle proteins, actin and myosin. The results of recent *in vitro* experiments have been interpreted to indicate that only myosin is the contractile protein, with actin playing an associated role, but not directly involved in the configurational changes resulting in contraction. Therefore, a series of experiments was done to test whether myosin alone was capable of contraction, without actin.

Purified myosin was prepared, and by viscosity tests and solubility tests shown to contain less than 0.1% actin. Pellicular fibers formed of this material at pH 7.0, and 0.05 *M* KCl, failed to develop tension upon addition of ATP, either at pH 7.6 or 9.0. Reconstituted actomyosin combining graded amounts of actin and myosin, developed graded tensions at pH 7.6, whereas at pH 9.0, there was no contraction. The graded tensions developed corresponded roughly to the "activity" values obtained from viscosity tests, which are in turn proportional to the amount of actin present. Isotonic contractions corroborated the findings obtained from experiments done under isometric conditions. Myosin fibers, formed at low temperatures with ATP also applied at low temperature, failed to develop tension when the temperature was raised, whereas actomyosin fibers treated similarly readily developed tension with the increase in temperature.

Implications as to the role of actin will be discussed. It is concluded that the contraction involves actin specificially and directly, and that myosin alone is not capable of contraction.

An interpretation of the action of certain chemical agents used in cancer therapy.[1] L. V. HEILBRUNN AND WALTER L. WILSON.

In spite of the vast literature on the chemotherapy of cancer, but little is known as to why various agents act as they do, and there has scarcely been even a hypothesis proposed as to why some agents which have a carcinostatic action may also be carcinogenic. We have studied the effect of nitrogen mustard, Nitromin, 6-mercaptopurine and urethane on eggs of the marine worm Chaetopterus. All of these substances have been used in the treatment of cancer. In dilute solution, all of them prevent cell division, and all of them act by suppressing the mitotic gelation. In more concentrated solutions, nitrogen mustard, Nitromin and urethane have the opposite effect, that is to say they tend to induce protoplasmic clotting. Thus they might well act to initiate mitosis, for the initiation of mitosis is caused primarily by agents which cause protoplasmic clotting. Indeed it can be shown that solutions of urethane do actually induce mitosis in Chaetopterus eggs. These facts are in line with the colloidal theories discussed in some detail in Heilbrunn's Dynamics of Living Protoplasm, published earlier this year by the Academic Press. Fractions of cow ovary extracts obtained by cold alcohol fractionation were also tested on Chaetopterus eggs. Fractions which prevent mitotic gelation act as rather powerful carcinostatic agents. This has been established by other members of our group who are now working with Ehrlich ascites tumors in mice.

A striking behavioral change leading to the formation of extensive aggregations in a population of Nassarius obsoletus.[2] CHARLES E. JENNER.

The distribution of *Nassarius obsoletus* in a given locality frequently presents problems of considerable ecological interest. Snails are sometimes absent from apparently favorable areas, although present in enormous numbers in adjacent but similar areas. The factors underlying this extreme patchiness in distribution are not readily apparent.

The problem was illustrated by a striking change in distribution pattern which occurred in a population of snails in Barnstable Harbor during the summer of 1956. The area under observation was an extensive mud-sand flat, approximately 200 × 800 yards, exposed only at low tide. In a well-populated area a reference zone was staked, marking 10 adjacent quadrats, 10 × 10 yards. During visits on June 19, July 1, 7, 15 and 22, snails were present in great numbers but were widely distributed over most of the flat. Observations during a brief visit on July 30 were hampered because the flat was not exposed at the time. It was apparent, however, that a definite change in snail distribution had taken place since the last visit; snails could be found in only one of the 10 quadrats. During the next observation (August 4), snails were not to be found over most of the flat and none were present in the marked area. The snails occurred chiefly in massive aggregations; in many areas they literally covered the ground, in some cases two or three layers deep. No snails were within 180 feet of the marked area, indicating that most had traveled more than this distance during the 13 days since the July 22 observation.

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[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

The functional significance of such mass migration and aggregation is not readily understandable. In the formation of the aggregations it seems clear that the animals were not responding independently to inanimate factors. Some type of interaction between snails must have been involved.

The timing of reproductive cessation in geographically separated populations of Nassarius obsoletus.[1] CHARLES E. JENNER.

The termination of seasonal reproductive activity was followed in populations of *Nassarius obsoletus* from Beaufort, N. C.; Great Pond, Mass. (south Cape Cod); and Barnstable Harbor, Mass. (north Cape Cod) during the spring and summer of 1956. In males of *N. obsoletus* the copulatory organ is resorbed at the end of the reproductive season (Jenner and Chamberlain, 1955), and the state of reproductive activity was judged by recording the per cent of unparasitized males bearing this structure. The per cent of unparasitized females having a formed egg case in the reproductive duct was also recorded.

For a given population cessation was found to be abrupt, with the major part of the transition period being only two to three weeks in duration. Curves for males and females were nearly superimposed. Reproductive activity was essentially completed for Beaufort snails by the end of May, for Great Pond by mid-July and for Barnstable by the latter part of August. The displacement of the curves for the different populations seems to be related to temperature, and agrees nicely with the known distribution of temperature along the Atlantic coast during the spring and summer months (Parr, 1933). The difference in timing, for example, between the north and south sides of Cape Cod (a latitudinal difference of 12 miles) was about as great as between south Cape Cod and Beaufort (latitudinal difference of over 500 miles). A May 17 collection from Mayport, Florida, indicated a timing of reproductive cessation similar to that of the Beaufort snails. Limited observations on snails from Boothbay Harbor, Maine, showed these snails to be two to three weeks later in their cycle than snails from Barnstable.

The method employed allowed a high degree of precision in the description of this seasonal event.

Absence of membrane potential in presence of asymmetrical ion distribution in the Fundulus egg. C. Y. KAO.

The accumulation of ions by biological membranes has generally been assumed to be accompanied by an electrical potential difference. In almost all excitable tissues at rest and in the egg of *Asterias forbesii,* the membrane potential approaches the value obtainable on the basis of the ratio of [K^+] on the two sides of the membrane. In the *Fundulus* egg, no membrane potential can be measured, in either the unactivated or activated state, across the plasma membrane, although a high membrane resistance is present. By means of flame spectrophotometry, potassium and sodium contents have been determined, using homogenized or aspirated egg material. K is present in 105 *mM*/liter of egg material in the unactivated egg, and 117 *mM* in the activated egg, whereas Na occurs in ca. 50 *mM*. The values for the corresponding ions in sea water are 10 *mM* and 452 *mM,* respectively. In addition to a lack of membrane potential, membrane resistance of activated egg is not materially affected by immersion in isotonic chloride solutions of Na^+, K^+ (540 *mM* each), Ca^{++}, and Mg^{++} (370 *mM* each) for up to 10 min. The discordance between the concentrations of the two major cations and the electrical properties of the egg plasma membrane should preclude generalizations with respect to the origin of membrane potentials in biological material.

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[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

Electron microscopic observations on the development of the chorion of Fundulus. NORMAN E. KEMP AND MARGARET D. ALLEN.

Pieces of ovaries containing developing oocytes were fixed in buffered (pH 7.4) 1% osmic acid in artificial sea water, embedded in methacrylate and sectioned with a Porter-Blum microtome set to cut at 0.025 μ. Sections reveal that cells of the follicular epithelium pull away from the oocyte as deposition of the zona radiata begins. The zona is a fibrous membrane perforated by so-called pore canals from the time of its first appearance. It thickens chiefly by the addition of new material on its inner surface. The pore canals, which account for the striated appearance of the zona radiata with the light microsocpe, contain microvilli, spirally coiled in older oocytes, extending from the surface of the oocyte outward to the subfollicular space. Branching protoplasmic processes of the epithelial cells are in close contact with, or possibly continuous with, the microvilli in the subfollicular space. Among the follicular cell processes are extracellular strands or filaments. The chorion of the ovulated egg is composed of (1) an internal portion, the chorion internum (Shanklin and Armstrong, 1952), consisting of lamellae arranged in a herringbone pattern, and (2) an outer portion, the chorion externum, consisting of tangentially oriented filaments in an adhesive matrix. The chorion internum is the transformed zona radiata of the oocyte; and the chorion externum is derived from the filaments and intercellular matrix of the follicular cell processes. We conclude that the chorion is anatomically a vitelline membrane (lamellated portion) combined with a true chorion (filamentous portion).

The nature of the metal-to-protein bond in hemerythrin. IRVING M. KLOTZ AND THEMIS A. KLOTZ.[1]

Hemerythrin, an oxygen-carrying pigment found in sipunculid worms, contains iron at its active sites but is devoid of porphyrin groups. Thus the metal seems to be attached directly to the protein. On a stoichiometric basis each active site, holding one O_2 molecule, corresponds to 2.4 Fe atoms.

Displacement experiments with other metal ions suggested that the iron is attached to sulfide groups of the protein. Titrations for mercaptan groups were carried out, therefore, with silver ion. The color of the oxyhemerythrin disappeared as silver was added and at the amperometric end point 2 Ag had been taken up for each 2.4 Fe in the protein. It would seem, therefore, that 2 of each 2.4 irons are attached to mercaptan side chains. Confirmation that the silver really displaces iron from a sulfide bond was obtained from quantitative studies of the reaction of oxyhemerythrin with a disulfide dye. The dye should react specifically with protein —SH groups; colorimetric titration agreed well with the silver amperometric titration.

These observations suggest that each active site of hemerythrin contains two iron atoms attached to the protein through cysteine side chains, the iron atoms holding an O_2 molecule between them. This configuration would also conform with previous results on the valence changes in the metal during the oxygenation process as well as with the new observation that approximately one-half as much mercury-(II) as silver-(I) combines with hemerythrin in amperometric titrations.

The molecular weight of hemoglobin from Petromyzon marinus. P. GALEN LENHERT,[2] WARNER E. LOVE AND FRANCIS D. CARLSON.

The present study was undertaken as a preliminary to a proposed x-ray diffraction analysis of this substance. An accurate molecular weight of Petromyzon hemoglobin was desired before attempting crystallization.

The sedimentation coefficient corrected to water at 20° C. was found to be 1.9 S units. A molecular weight of 23,600 was calculated from data taken during the approach to sedimentation equilibrium by the method of Archibald as modified by Klainer and Kegeles. A value of 0.751 given by Svedberg and Eriksson-Quensel as the partial specific volume of Petromyzon hemoglobin was used in this calculation. This figure must be considered approximate due to our inability to obtain optimal photographic conditions.

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[1] This investigation was supported in part by a research grant, RG-4134, from the National Institutes of Health, Public Health Service.

[2] National Science Foundation Fellow 1955–56.

Iron was determined by Lorber's sulfosalisylic acid method. The values obtained, using an extinction coefficient for the iron-sulfosalisylic acid complex of 0.101 per microgram of iron per mililiter, indicate one mole of iron for every 17,800 grams of protein. Heme was measured as the reduced pyridine hemochromogen, giving a value of 18,600 grams of protein per mole of heme.

These results in conjunction with the sedimentation equilibrium molecular weight given above support the conclusion that there is one heme group per molecule of hemoglobin. The molecular weight which results from these data is 18,200 ± 400. This result of approximately 20,000 is in agreement with Svedberg's values for other cyclostome hemoglobins. Attempts are now being made to crystallize this protein.

Hyaline polymer of the fertilized egg of Arbacia punctulata. Arthur K. Parpart and Julien Cagle.

The hyaline layer which forms during the first 5 to 10 minutes after fertilization of the egg of *Arbacia punctulata* is the resultant of a polymerization of a polysaccharide probably released as a monomer upon the explosive breakdown of the cortical granules. The hyaline layer can be depolymerized completely and rapidly, 30 seconds, in isosmotic solutions of non-penetrating non-electrolytes (*e.g.,* glucose, xylose, erythritol, glycerol), and partly depolymerized in the presence of (a) 0.5 M NaCl, (b) 0.5 M NaCl plus 0.01 M $CaCl_2$, and (c) 1.0 M glucose plus either 0.1 M guanidine HCl or 0.001 M $CuCl_2$. Since guanidine and copper are active protein precipitants it is suggested that the hyaline layer is primarily composed of a polymerized polysaccharide which can be readily depolymerized. Upon depolymerization this polysaccharide exerts an osmotic pressure equivalent to ca. 5% egg albumin, which draws water into the perivitelline space. A depolymerized hyaline layer can be repolymerized by means of magnesium ions (0.005 M to 0.04 M) dissolved in isosmotic glucose, and an optically refractile and dense hyaline layer is re-formed. Upon repolymerization the colloid osmotic pressure in the perivitelline space decreases.

Magnifying the invisible. Delbert E. Philpott and George G. Lower.

A 16 mm. color movie has been made to show the basic techniques used in electron microscopy. An overall view of the R.C.A. Model EMU-2B and the North American Phillips Company Model EM-100 is shown. Specimen grids are prepared, and the complete process of a preparation of liquid suspension of virus is demonstrated. The shadow casting machine and technique are illustrated. To show how living tissues are prepared for the electron microscope, a frog heart is taken through the steps of fixation, embedding, and sectioning. Actual operation of the microscope is also shown. Various pictures of different tissues and specimens taken with the microscope are shown to demonstrate the adaptability of the electron microscope to a variety of research problems. A wave-length chart shows the difference between light and electrons as a source for microscopy, and a comparison of magnification sizes is demonstrated.

This film is meant to be an introduction to electron microscopy for the layman, to clarify to those who have never had the occasion to work with one, the basic principles and techniques used.

Sodium ion exchange in Ulva lactuca. George T. Scott, Robert DeVoe and Gary Craven.

Sodium ion was observed to exhibit an unexpectedly high rate of turn-over between the cells of *Ulva lactuca* and the surrounding sea water. This alga is a very favorable organism for the study of ion exchange since it consists of but two layers of cells. Furthermore, extracellular fluid with contained electrolytes can be removed by the combination of a thirty-second rinse in isotonic sucrose and a triple blotting technique in absorbent tissue. Previously published data indicate that sodium and potassium ions diffuse into isotonic sucrose from the extra-cellular phases within three seconds.

The exchange experiments involved placing the alga for varying lengths of time in sea water containing 0.5 to 1.0 millicurie of Na^{24} per liter followed by the thirty-second sucrose rinse and the triple blotting procedure. The samples of algae consisted of small discs about two inches in diameter cut from a single large frond. Activities were determined, following which the samples were wet ashed and analyzed for sodium by flame photometry; specific activities were then calculated. Time course curves reveal that at twenty degrees the specific activities of the algae had reached equality with that of the sea water within ten seconds. At one degree exchange was nearly complete in fifteen seconds. Experiments done at twenty degrees, in which the sea water contained 1×10^{-3} M phenylurethane in one case, and in another 5×10^{-4} uranyl nitrate at pH 4.8, all in addition to the Na^{24}, showed a slight lessening of the exchange rate, the significance of which is in doubt. Uranyl ion has been shown to be preferentially surface adsorbed.

The data suggest, because of the very high rate of sodium turnover, an exchange of sodium ion between the surface of peripheral zone of the cell and the medium; this exchange probably does not directly involve an active metabolic pump *per se.*

Studies on parasites of the green crab, Carcinides maenas. Horace W. Stunkard.

Since *Carcinides maenas* is a serious predator of *Mya arenaria*, the U. S. Fish and Wildlife Service is interested in determining whether or not its parasites can serve as possible means of biological control. It has long been known that *C. maenas* in the Woods Hole area is infected by an undetermined, encysted metacercaria and experiments have been conducted to discover its identity, life-history and biology. Metacercariae were excysted to study their morphology and cysts were fed to recently hatched, uninfected birds, *Sterna hirundo* and *Larus argentatus.* Large numbers of worms were recovered, including all stages from juvenile to fully mature specimens. The structure of the metacercariae suggested that they may be specifically identical with a minute, stylet-bearing cercaria which occurs in *Littorina obtusata, Littorina saxatilis,* and rarely in *Littorina littorea.* Small green crabs exposed to these cercariae became heavily infected; enormous numbers of worms entered the tissues and developed to metacercariae, identical with those of the natural infections mentioned above. Small crabs exposed continuously with six to eight infected snails died in ten to twenty days, and on dissection yielded thousands of larvae. The stages in the life-cycle of the parasite agree with descriptions by European investigators of corresponding stages: the metacercariae with metacercariae from *C. maenas*, described but not named by M'Intosh (1865); the adults with *Microphallus similis* from Swedish gulls, described and named by Jägerskiöld (1900); and the cercariae with *Cercaria ubiquita* Lebour, 1907. The identity of these worms and their relation as stages in the life-cycle of a single species is predicated. It is possible that the parasites and their intermediate hosts are introduced species.

Conduction velocity in the giant axon of the squid (Loligo pealii) in D_2O. Roger E. Thies[1] and Francis D. Carlson.

A partially cleaned axon was threaded into a glass chamber with a volume approximately thirty times that of the treated region of the nerve. A few centimeters of the nerve were treated with a D_2O sea water (pH 8) made by evaporating artificial sea water to dryness and bringing the residue up to volume with 99.5% D_2O. A constant temperature bath maintained the preparation at $17.0 \pm 0.5°$ C. The stimulus was applied to an untreated region. The diphasic action potential was recorded within each end of the treated region, and both action potentials were displayed on the same single sweep of an oscilloscope. Measurement of the distance between the peaks of the action potentials gave the value of the conduction time to within ± 2%.

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[1] National Science Foundation Fellow, 1956–57.

In eight experiments with five nerves the conduction velocity decreased by 19 ± 3% after treatment with the D_2O solution. This agrees with the finding of Garby and Nordquist on similarly treated frog sciatic nerve. Recovery in ordinary water solutions was to within 95 ± 4% of the original value. Both the initial decrease in velocity and the recovery were complete within three to five minutes. Lower concentrations of D_2O produced smaller decreases in the conduction velocity, the dependence being approximately linear. The effect is not due to small pH differences, since between pH 6.1 and 9.7 the conduction velocity remained unchanged to within ± 3%. The D_2O produced no detectable change in the diameter of the axon.

The small magnitude of the effect suggests that D_2O reduces conduction velocity because its viscosity is 1.23 times that of ordinary water, and ionic mobilities are correspondingly reduced. Further experiments are required to substantiate this hypothesis.

Change in rate of release of K^{42} upon fertilization in eggs of Arbacia punctulata. ALBERT TYLER [1] AND ALBERTO MONROY.[2]

From previous experiments demonstrating the existence of an electrical potential difference across the surface of echinoderm eggs, a rapid decrease followed by an increase of this potential upon fertilization, reversible depolarization by addition of K^+ externally, and a much greater concentration of K^+ inside the egg than in the surrounding sea water, it appeared that the process of fertilization has features in common with the stimulation of other excitable tissue such as muscle and nerve.

This leads to the expectation that there should be an increased rate of exchange of K^+ between the inside and outside of the egg upon fertilization.

By loading eggs of Arbacia with K^{42}, washing them, fertilizing aliquots and determining radioactivity of the supernatant, it has now been found that the rate of release of the K^{42} increases very markedly (1.5 to 3 X) upon fertilization. The results of seven experiments were consistent in showing that the increased rate of release starts within 1½ to 2 minutes after fertilization. There is also consistently a reduction of rate during the 5th to 8th minute, followed again by a rise.

The reversible replacement of potassium by rubidium ion in Ulva lactuca. ROBERT DEVOE, GEORGE T. SCOTT AND GARY CRAVEN.

Pieces cut from fronds of *Ulva lactuca* were placed in an artificial sea water containing rubidium instead of potassium ion, and samples harvested in triplicate at various times. After ninety-six hours the remaining pieces were placed in running sea water and samples harvested up to one hundred and twenty hours more. All samples were rinsed in isotonic sucrose solution and triple-blotted with absorbent tissue to remove all extracellular electrolytes, then ashed and analyzed for potassium, rubidium and sodium ion by flame photometry.

The uptake of rubidium was rapid and complete with four hours, the time the first samples were taken, and amounted to 65% of the original potassium present. The potassium ion concentration, after an initial drop to 40% of its original value, continued to decrease slowly in a manner parallel to the potassium ion concentration in the control. After ninety-six hours, when the samples were placed in running sea water, the gain in potassium and the loss of rubidium were rapid for the first ten hours, then more gradual. After one hundred and twenty hours in running sea water, the potassium ion concentration had practically reached that of the control, but the rubidium ion concentration had dropped to only 30% of its highest value. Sodium ion concentration was relatively constant during the course of the experiment.

Ulva lactuca does not show a time lag due to adaptation to rubidium as do some other organisms. It does photosynthesize when containing rubidium, and will form and discharge a germinal ridge.

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[1] Supported by research grant C-2302 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[2] Supported by a grant from Consiglio Nazionale delle Ricerche.

PAPERS READ BY TITLE

Observations on autotomy in the starfish, Asterias forbesi. John Maxwell Anderson.

Autotomy of rays in *Asterias* has excited the interest of many investigators but has never been adequately analyzed, apparently because attention has been focussed chiefly on subsequent processes of regeneration. Autotomy normally follows severe injury to a ray and can be artificially induced by application of electric current (8–16 V DC). As King (1898) reported, separation always occurs at one side or the other of a specific pair of ambulacral ossicles and proceeds rapidly at this level around the body-wall. The logical supposition that separation involves muscular contraction is verified by the fact that it is prevented by treatment of the animal with isotonic $MgCl_2$ solution. Under this narcosis, the animal can be subjected to radical operations, such as complete removal of its aboral body-wall; replacement in sea water and recovery from narcosis do not then evoke spontaneous autotomy. Electrically induced breakage in the floor of a ray thus exposed involves a sudden release of attachments binding the 5th pair of ambulacral ossicles to its neighbors; these ossicles, bound together by transverse muscular and collagenous fibers and bearing a pair of tube feet, can be lifted out with forceps after being thus released. Sections of these elements reveal that transverse connections are intact, but that the longitudinal muscle bands and connective tissues have been torn across. Initiation of autotomy does not necessarily depend upon this separation of ambulacral elements; it can be induced by electrical stimulation in the isolated aboral body-wall, where softening and tearing of muscular and connective tissues occur along a predetermined line. This cannot be regarded as simply a level of physical weakness, as attempts forcibly to break off a ray do not result in separation at this point. Serial sections of rays have shown no recognizable structural peculiarities of skeletal, muscular, or nervous elements in the region of the breaking-joint.

The innervation of muscle fibers in the extrinsic stomach-retractor strands of the starfish, Asterias forbesi. John Maxwell Anderson.

The extrinsic retractor strands are muscular bands extending in pairs from fibrous nodules on the outer wall of the cardiac stomach to attachments on the ambulacral ossicles in the floor of each ray. Their position raises a question as to the source of innervation for their muscular elements, whether by fiber tracts originating in the subepithelial nerve plexus layer of the gut wall or by pathways originating in the radial nerve cord of the ray. Vital staining procedures, utilizing leucomethylene blue, demonstrate that several groups of neurons traverse the mesentery-like sheets binding the strands to the floor of the ray, penetrate the strands, and terminate in typical asteroid "ribbon axons" embracing the muscle fibers. While it has been impossible to trace precisely the origin of these neurons, their courses make it highly probable that they arise, along with nerve fibers to other sets of muscles in the ray, from lateral motor centers similar to those described for *Astropecten* by J. E. Smith (1950). No fibers have been found crossing the nodule from the wall of the stomach. It may thus be concluded that the coördinated action of the retractor muscles and of the muscles in the wall of the stomach does not depend on common pathways of innervation.

The effects of x-irradiation on the pupae of the yellow mealworm, Tenebrio molitor Linn. Alan Priest Brockway.

In these experiments a genetically mixed culture of larvae was maintained at room temperature on a mixture of white flour and dried brewers yeast. Each morning the culture was cleared of pupae and then every four hours the pupae were collected and irradiated. Doses ranging from 500 r to 20,000 r were given at the rate of 2,500 r per minute.

All pupae used as controls or given 500 r and 1,000 r hatched normally. Of those given 2,000 r only 33% hatched normally. All pupae given 2,500 r to 20,000 r hatched abnormally. The region between 1,000 r and 2,500 r seemed to be quite critical. There was also an effect on the length of the pupal stage. The average length of pupation for the controls was 8.15 days whereas that of the pupae given 500 r was 9.0 days, 1,000 r to 4,000 r was 10.0 days and 5,000 r to 20,000 r was 11 days. Furthermore at doses from 5,000 r to 20,000 r only 50% of the pupae hatched. Work is being carried on currently to determine the radiosensitivity of the pupae at ages other than within four hours of formation.

At 2,500 r the emerging adult was unable to shed the pupal cuticle, which remained in a mass on the tip of the abdomen. An increase of 500 r (3000 r) inhibited the shedding of the cuticle from most areas of the body. This was quite noticeable on the elytra and wings and even if the cuticle was removed with forceps these structures did not expand. The tanning process of the new cuticle was not complete in any of the adults given more than 3,000 r in the pupal stage. Only some of the cuticle was tanned giving the animal a mottled appearance and many soft areas. At times there also appeared blisters on the elytra and wings. It appeared that in the elytra the epidermal gland poured down an excess of cuticlin. The blisters on the wings were filled with a fluid which may be hemolymph, though there have been observed no hemocytes in the fluid.

Chromatographic study of crystalline style amylase.[1] ALFRED B. CHAET.

During the course of an investigation it became necessary to determine whether the amylase activity observed in the crystalline style of *Mya arenaria* was due to α-amylase, β-amylase, or a mixture of the two. The technique used in this study was that of paper chromatography followed by incubation with the enzyme's substrate. Descending type chromatograms were run in the dark at 18 ± 1° C. on strips of Whatman No. 1 filter paper. After the front had descended a certain distance, the paper was air dried and placed face down in close contact with an agar-substrate film. This substrate film was prepared by allowing a 4% solution of agar (buffered at pH 7) containing 2% potato starch to gel on a large glass plate. The chromatogram-substrate system was sealed and incubated for 36 hours at 30° C. The position of α- and β-amylase was observed after removing the chromatogram and spraying (either paper or agar-substrate sheet) with a dilute solution of iodine-potassium iodide. Two satisfactory solvents were found for distinguishing between α- and β-amylase. It was shown, using a mixture of ether and phosphate buffer (pH 6.6), that α-amylase has a R_f. of 0.66, whereas β-amylase exhibits no activity. When using a 38% saturated solution of $(NH_4)_2SO_4$ as the solvent, α-amylase had a R_f. value of 0.02 and β-amylase, 0.27. Although in both solvents the spots are elongated, there is no difficulty in distinguishing the difference between the two enzymes. Experimental analysis of Mya crystalline styles shows that all of the amylolytic activity previously observed is due only to α-amylase.

Mechanism of toxic factor release.[2] ALFRED B. CHAET.

Additional experiments were carried out dealing with the characteristics of the toxic substance obtained from scalded worms (*Phascolosoma gouldii*) and an attempt made to study the mechanism of toxin release. The results indicate that in scalding experiments heat *per se* does not produce the toxin by changing a non-toxic molecule into a toxic one, for when suspensions of washed cells taken from the coelomic fluid are ruptured (by homogenization) in the absence of heat a toxin is released which, when injected into normal worms, causes death in 62 hours. This toxin has the same characteristics previously reported for the toxic factor extracted from scalded worms. It is a heat-stable, non-dialyzable molecule which precipitates in saturated $(NH_4)_2SO_4$. It appears from these and other experiments that the toxic factor is normally present in the form of a toxic molecule found within the coelomic fluid cells and that rupturing the cell by heat, crushing, or hypotonic solutions is sufficient to merely release this substance which, when allowed to circulate through the rest of the organism, results in death. When using *Limulus* as a test object it has been shown than intravenous injection of from 0.002–0.003 ml. of toxin per gram of *Limulus* is sufficient to cause death in less than 24 hours. This test object may prove useful in studying the mode of action of the toxic factor. The stability of the toxin is illustrated in experiments whereby samples stored in a frozen state for 10 months showed no apparent loss of biological activity. By differential centrifugation experiments it was possible to separate the coelomic fluid cells into 5 distinct fractions, but no single fraction was the storehouse for the toxic factor since at least 4 out of 5 released the toxic material when heated *in vitro.*

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[1] Supported by funds from the State of Maine, Dept. of Sea and Shore Fisheries.

[2] Supported in part by funds from Boston University School of Medicine and Coe Research Fund.

A combined effect of urea and borate buffer on uricase activity.[1] AURIN M. CHASE.

The effect of urea and borate buffer concentrations on the oxidation of uric acid by uricase was studied by measuring the change in extinction at 300 mμ using the Beckman DU spectrophotometer, maintained at 26° C. by water cooling. Reaction mixtures contained 0.33 mg./ml. of uricase (Worthington crude preparation) and 8 μg/ml. of uric acid, plus the desired urea concentration; all dissolved in pH 9 borate buffer of the required molarity. These molarities were 0.10, 0.23, 0.37, and 0.50; each used in conjunction with urea concentrations up to 9.0 *M*. After adding uricase the extinction was measured for 10 minutes, during which time the reaction was zero order. The slope of the resulting line was taken as the measure of enzyme activity.

Borate buffer by itself had an inhibitory effect on uricase activity, the initial rate in 0.5 *M* being about 75% that in 0.1 *M* concentration. With 4.8 *M* urea present, activity in 0.5 *M* borate was about half that in 0.1 *M*. In 0.5 *M* buffer regular, increasing inhibition occurred with increasing urea concentrations whereas, in 0.1 *M* buffer, there was no inhibition until nearly 4.0 *M* urea concentration was reached. Then activity dropped to zero as 8.0 *M* urea concentration was approached. The intermediate borate concentrations gave intermediate results.

Uricase inactivation at the lower urea concentrations was essentially instantaneous. Above 6.4 *M*, however, a slower inactivation occurred in addition to the rapid effect. The pH of all four buffers was increased from 9.0 to 9.7 by added urea up to 9.0 *M*, and this may well be a complicating factor.

It is known (Canellakis and Cohen, 1955) that intermediates and end products of this reaction may differ in different buffers. The present results show, in addition, that the kinetics observed for urea inactivation can be considerably influenced by the concentration of the buffer used.

Dimethylated dioxypurines and/or x-ray inhibition of Arbacia egg development. RALPH HOLT CHENEY.

Current dimethylxanthine and x-ray inter-relationship studies regarding their inhibitory action, separately and combined, on growth phenomena were done using the *Arbacia punctulata* egg as the test material. *M*/400 theophylline (1:3 CH_3 2:6 dioxypurine) and *M*/400 theobromine (3:7 CH_3 2:6 dioxypurine) and 30,000 r x-ray dosage were employed for comparison with reports (Cheney 1948, 1949) on inhibition by Tp, Tb, and caffeine (1:3:7 CH_3 2:6 dioxypurine); upon caffeine with x-ray (Cheney and Rugh 1954); and the report by Cheney (1955) on purine ($C_5H_4N_4$). Tb is somewhat more inhibitory than Tp at *M*/400. 30,000 r delays but does not arrest egg development prior to the stage at which *M*/400 of either drug separately stops growth without irradiation. 30,000 r does not affect the fertilizing power of the sperm. Eggs were x-rayed before fertilization.

Development of non-irradiated eggs, x-irradiated with or without drug; drug-treated 30 minutes with or without x-rays; also, immediate mixing for insemination in drug without pretreatment, was observed until controls reached pluteus. Subsequent to experimental conditions cited as prior to mixing with normal sperm, equivalent egg numbers were transferred to stender dishes, inseminated by normal sperm, and maintained in running sea water (SW). Developmental time and form were recorded in SW, SW with drug, irradiated SW without drug, and irradiated SW with drug.

The over-all effect of x-irradiation alone, *M*/400 Tb and Tp with and without 30,000 r was delay in development: first, at prophase amphiaster; second, at blastula-gastrula sequence; and third, to eventually arrest development. No evidence was found to indicate any significant synergistic, summation, nor antagonistic action between either Tb or Tp and 30,000 r irradiation although each of the three factors is separately inhibitory. This suggests that their individual effects are via different mechanisms.

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[1] Supported in part by a National Science Foundation grant.

The distribution of mitochondria and lipid droplets during early cleavage in Ilyanassa obsoleta. A. C. CLEMENT AND F. E. LEHMANN.

Mitochondria were identified by their size and affinity for Janus green. In centrifuged eggs they form a band between two layers of clear cytoplasm. The Janus green stain of the mitochondrial band may be fixed with OsO_4. The normal localization of mitochondria and lipid droplets has been followed from the one-cell stage through the time of formation of the fourth quartet of micromeres. The position of mitochondria was revealed by vital staining with Janus green (1:100,000 solution), and that of lipid droplets by staining with Sudan III or OsO_4.

Before cleavage the mitochondria are concentrated mainly in a broad cytoplasmic cap around the animal pole. Each of the first 4 blastomeres shows abundant mitochondria. All of the cells of the first three quartets of micromeres, and their derivatives, also receive abundant mitochondria. After the formation of the first and second micromere quartets, the mitochondria in the macromeres are massed in a broad crescentic zone where the cytoplasmic cap meets the yolk zone. The mesentoblast cell 4d receives numerous mitochondria; the fourth quarter entoblasts (4a, b, c) also contain numerous mitochondria, whereas the 4A, B, C and D macromeres show relatively few.

Lipid droplets are concentrated near the animal pole of the uncleaved egg and are apportioned rather evenly to the first 4 blastomeres. In the early macromeres the lipids form a crescentic band between the cytoplasmic and yolk zones. In all of these respects the general pattern of lipid distribution is rather similar to that of the mitochondria. However, a gradient of lipid distribution appears during the formation of the micromere quartets. The first quartet micromeres receive very few lipid droplets, the second somewhat more and the third still more. The 4d cell receives nearly all of the lipids from the 3D macromere; there is a more even distribution of lipids at the subdivision of 3A–3C.

The uptake and distribution of radioactive alloxan in islet and other tissues of the toadfish. S. J. COOPERSTEIN, ARNOLD LAZAROW AND WILMA LAUFER.

As part of a broad research program on the problem of diabetes mellitus we have been interested in determining the mechanism by which alloxan selectively kills the insulin-producing cells of the islets of Langerhans and thereby produces diabetes. One of the immediate problems in elucidating the mechanism of alloxan action is to determine whether this chemical agent is selectively concentrated by the insulin-producing beta cells or whether the selectivity of alloxan for the beta cells is due to their specialization for insulin synthesis. The toadfish is ideally suited for these studies. Whereas in mammals the islet tissue is distributed throughout the pancreas and constitutes only 1% of its total weight, in the toadfish the islet tissue is segregated into a discrete mass which is separated from the pancreatic acinar tissue. Therefore we have studied the uptake of radioactive alloxan by the principal islet and other tissues of the toadfish, *Opsanus tau.*

We have developed suitable techniques for injecting alloxan directly into the circulation through a gill arch vessel. In this manner we have determined the distribution of radioactive alloxan in the various tissues as early as 90 seconds after injection and at various later times (2, 2 and ½, 5, 15 and 30 minutes). The absolute amount of radioactivity found in the various tissues increased rapidly and then gradually decreased with time. Its relative distribution among the different tissues, however, did not change significantly. Blood had the highest activity at all times. The activity in the other organs decreased in the following order: gill, heart, kidney, islet, brain, liver and skeletal muscle. The islet tissue had only ¼ to ⅕ of the radioactivity found in blood, and at no time did its radioactivity exceed that which would have resulted from uniform distribution of the radioactive alloxan throughout the body.

Phosphorylase system in the lobster.[1] ROBERT W. COWGILL.

Phosphorylase catalyzes the reversible formation of glycogen and inorganic phosphate from glucose-1-phosphate. This enzyme exists in tail muscle of the lobster in three forms: phosphorylase a is active enzymatically, phosphorylase b requires muscle adenylic acid for activity, and phosphorylase c is completely inactive. The relative levels of these forms of the enzyme change with the physiological condition of the animal (Cowgill and Cori, 1955) but the principal form extracted from the normal lobster is phosphorylase c.

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[1] This work was supported by a grant from the National Institutes of Health.

Phosphorylase c has been separated from phosphorylase a and phosphorylase b by ammonium sulfate fractionation of crude muscle extracts. It has been tested in the presence of a wide variety of metal ions and nucleotides and it has been found completely inactive. The ammonium sulfate fractions also contain enzymes for the interconversion of the various forms of phosphorylase. Two of these are activating enzymes. Activating enzyme No. 1 converts phosphorylase c to phosphorylase b; this enzyme requires Fe^{+++}, Cd^{++}, Pb^{++}, or VO^{++} for activity. Activating enzyme No. 2 converts phosphorylase b to phosphorylase a, and requires Mn^{++} and adenosine triphosphate for activity. Both the metal ion and nucleotide requirement are highly specific for the latter enzyme. In addition to these enzymes, there is at least one inactivating enzyme that converts phosphorylase a to phosphorylase c. This enzyme requires Mn^{++} or certain other divalent cations for activity, but unlike the second activating enzyme it is completely inhibited by fluoride ions. All of the activating and inactivating enzymes were inhibited by ethylenediaminetetraacetic acid.

It is interesting that these enzymes that interconvert the various forms of lobster phosphorylase also are capable of interconverting phosphorylase a and phosphorylase b from rabbit muscle. That is, the inactivating enzyme of lobster will convert rabbit phosphorylase a to phosphorylase b (but not to a phosphorylase c form); and activating enzyme No. 2 of lobster will convert rabbit phosphorylase b to phosphorylase a.

The action of Nessler's reagent and ATP on extracted and denatured muscle.[1] DAVID DIBBELL AND HOWARD HOLTZER.[2]

The following experiments suggest that Nessler's reagent has more than one mode of action on the muscle model; one simulates the action of ATP, the other does not.

Muscle fibers contracted maximally in either ATP or Nessler's and inspected under phase, exhibited extreme contraction bands. Such contracted fibers may be stretched to, and maintained at, 300% of rest length by treatment in Nessler's followed by versine (Lacki). Stretched fibers can be recontracted by Nessler's but not by ATP. If a contracted fiber is stretched an expanded contraction band pattern is observed. Subsequent changes in length do not change this contraction band pattern. Fibers lightly prefixed in formalin to inhibit contraction and extended in Nessler's, yield the stretched A, I, Z and H band pattern. Thus ATP or Nessler's induced maximal contractions produce the same irreversible submicroscopic reorganization which is expressed cytologically as contraction bands.

Differences between ATP and Nessler's were demonstrated by treating muscle models with heat, formalin, acetone, absolute alcohol, low pH, and extracting in 0.5 *M* KI, 0.9 *M* LiCL or urea. In all these instances, Nessler's induced contractions after the muscle proved refractory to ATP. For example, 0.5 *M* KI extracted fibers do not contract to ATP after one hour, but react to Nessler's after 6 hours. Extension in Nessler's followed by contraction can be observed in treated fibers that will not contract from rest length. For example, fibers extracted in 0.5 *M* KI for two weeks though not contracting when initially placed in Nessler's, when stretched, contract vigorously. Nessler's will contract living muscle, whereas ATP will not. These experiments indicate that Nessler's, in addition to its effect on the ATP-sensitive actomyosin complex, may act on the muscle fiber skeleton.

Fermentation studies in nine varieties of Tetrahymena pyriformis.[3] ALFRED M. ELLIOTT AND DARRYLL E. OUTKA.

In a search for biochemical differences in *T. pyriformis*, clones representing each of the 45 mating types of the nine known varieties were tested for their capacity to ferment the following sugars: dextrose, levulose, galactose, mannose, sucrose, maltose, and lactose. To the basic medium, containing 0.5% proteose peptone, 0.5% tryptone and phenol red as the indicator, filtered sugars were added aseptically to make up final concentrations of 0.5%. The pH was initially set at 7.2. Light inoculations (0.2 ml.) of the organisms in log growth were dispensed into the test and control media which were then incubated at 27° C. Records of color changes were made at one, two, three and 5 days; in doubtful cases the tubes were maintained for as long as 12 days.

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[1] Supported by Multiple Sclerosis Society and National Foundation for Infantile Paralysis grants.

[2] American Cancer Society Scholar.

[3] This investigation was supported by grants from the National Institutes of Health (PHS G3588C3) and the Horace H. Rackham School of Graduate Studies, University of Michigan.

All clones fermented dextrose, mannose, and maltose. Levulose was degraded by all except two clones in variety 1. None fermented sucrose and only four of the five clones tested in variety 9 attacked lactose. The widest variation occurred with galactose which was fermented by all clones in varieties 1, 3, 7 and 9 but was unaffected by clones from varieties 2, 4, 5, 6 and 8.

These results indicate considerable variation in fermenting capacity among the clones tested, and although varietal differences are striking, the number of clones tested is insufficient for any final generalizations. Only after a large number of clones from each variety are examined can general conclusions be drawn.

Electron microscope studies of conjugating Tetrahymena pyriformis.[1] ALFRED M. ELLIOTT AND JOHN W. TREMOR.

The sequence of events occurring during conjugation in *T. pyriformis* (strains WH6 and WH14, variety 1, mating types I and II) was observed under the electron microscope. Concentrated suspensions of washed cells in various stages of conjugation were prepared from both homogenized and sectioned material. The former proved unsatisfactory owing to destruction of nuclear elements. Sectioned material revealed certain details of conjugation not observed with the light microscope. The organisms were fixed in 1% veronal-acetate buffered formalin and treated with 2% osmic acid. They were then concentrated, embedded in methacrylate, and sectioned.

Sections through the region of contact between the two mates showed regularly spaced protoplasmic bridges of approximately 0.2 micron in diameter which could conceivably serve for the exchange of cytoplasmic materials. These were well established at the time of the third prezygotic division and possibly earlier. Since they possess about the same diameter and spacing as cilia, it is possible that they were derived from them. The morphology of the membranes in the region of nuclear exchange was clearly seen. Most of the nuclear stages occurring during conjugation were observed. Examination of early and late macronuclear anlagen stages revealed structure which could not be clearly interpreted from the preparations. Chromosomes were readily observed but showed nothing that had not already been seen under the light microscope. It is hoped that with better fixation other details may be seen that will supplement our knowledge of the morphology of this and other ciliates.

Influence of hematoporphyrin and phenol on x-radiation sensitivity of Paramecium. FRANK H. J. FIGGE[2] AND RALPH WICHTERMAN.[3]

In previous experiments, hematoporphyrin solutions containing phenol as a preservative had been employed to alter the radiation sensitivity of *Paramecium caudatum.* Paramecia placed in the solutions of hematoporphyrin prior to irradiation exhibited an LD 50 (24 hours) of 18 kr while the control groups required a dose of 340 kr. Experiments reported here were designed to ascertain the influence of phenol and hematoporphryin on radiation sensitivity when used independently and combined. The conditions of the radiation experiments were essentially the same as those described in our earlier publications. *Paramecium bursaria* and *P. multimicronucleatum* were irradiated in Nylon syringes so that samples could be taken at various dose intervals up to 100,000 r. The highest dose killed none of the controls. All of the animals placed in porphyrin and phenol combined were killed by 50 to 100 kr doses. The LD 50 for *P. bursaria* was 16 kr, the same as observed previously. The 1 : 20,000 hematoporphyrin solutions without phenol had relatively little observable effect since nearly all animals of both species survived the maximum dose given. Animals placed in 1 : 10,000 phenol solutions corresponding to the concentration of phenol in the porphyrin and phenol mixtures were sensitized to x-radiation. None of the animals survived a dose of 33 kr. The LD 50 for the phenol-treated group was even lower (14 kr, *P. bursaria*) than the solutions containing hematoporphyrin and phenol.

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[1] This investigation was supported by research grants from the National Institutes of Health (PHS G3588C3) and the Horace H. Rackham School of Graduate Studies, University of Michigan.

[2] Supported by grants from the Anna Fuller Fund and the American Cancer Society, Maryland division.

[3] Part of a project aided by a contract between the Office of Naval Research, Department of the Navy, and Temple University (NR 135-263) and the Committee on Research, Temple University.

It was also observed that the irradiated animals in the phenol solution survived much longer (12 hours) when kept in Nylon syringes and deprived of air while specimens that were expressed from syringes into spot plates died within 30 minutes.

Thus the increased sensitivity of paramecia is due mainly to the phenol which is used as a preservative for the porphyrin solutions. Attempts will be made to utilize phenol in combination with porphyrin and phenol alone in cancer therapy.

The effect of argon at high pressures on the cleavage time of the sea urchin, Arbacia punctulata. CHARLOTTE HAYWOOD.

Several investigators have shown that the inert gases, nitrogen and argon, at sufficiently high atmospheric pressures can exert a narcotic effect on certain tissues and on animals, including man. A previous study of nitrogen at pressures up to 61 atmospheres upon fertilized Arbacia eggs failed to demonstrate a delay in the cleavage rate. A similar study has now been made with argon since it might be expected to be more effective than nitrogen because of its greater lipid solubility.

The method is essentially the same as that used with nitrogen at high pressures (already published). A pressure chamber which permitted microscopic observation was employed. Adequate oxygen was available in the air initially present in the chamber. Controls in air at atmospheric pressure were run simultaneously. Temperatures were 21.5° to 22.0° C.

The time required for the first cleavage to appear in 50% of the eggs was regarded as the cleavage time. In ten experiments with argon at 41 atmospheres the cleavage rates were retarded 4 to 8 minutes (average = 5.9 minutes). These values represent delays of 8 to 15 per cent (average = 11%) beyond the control cleavage times. In six experiments at 55 atmospheres of argon the delays were 9½ to 18 minutes (average = 14.2 minutes), or cleavage times 16.4 to 34% (average = 26.2%) longer than the controls. At still higher pressures the delays were greater, although pressures up to 68 atmospheres failed to give complete suppression of cleavage. Abnormalities were frequent at the pressures above 55 atmospheres.

Hydrostatic pressure *per se* is not involved with 41 and 55 atmospheres at least, as shown by earlier negative results with nitrogen and with helium at 61 atmospheres.

The enhancement of somitic muscle maturation by the embryonic spinal cord.[1] HOWARD HOLTZER,[2] JAMES LASH AND SYBIL HOLTZER.

Recent experiments in this laboratory indicated that in the chick maturation of somitic muscles was enhanced by the spinal cord but unaffected or inhibited by the notochord. This relationship prompted a re-examination of the situation in amphibian embryos, where it has been claimed that the notochord is essential to somitic myogenesis. The following experiments will demonstrate that in salamander, as well as chick embryos, the spinal cord stimulates the growth of somitic muscle, whereas the notochord is inert.

Clusters of 6 to 10 isolated somites from tail-bud embryos were implanted into the dorsal fin of young larval hosts. At time of sacrifice three to 5 weeks later, small strands of muscle and pronephric tissue were found in the quasi-culture chamber of the dorsal fin of the host.

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[1] Supported by U.S.P.H. Service and Multiple Sclerosis Society grants.

[2] American Cancer Society Scholar.

Implanted clusters of somites plus notochord differentiated into a similar small mass of muscle. Irregular and inconstant mounds of cartilage adhering to the notochord and scattered kidney tubules were also present. In contrast, the same mass of somites plus a piece of spinal cord cultured in the dorsal fin formed a large mass of well differentiated muscle, similar to what the somites would have formed if left *in situ*. Cartilaginous vertebral elements, including precociously formed centra, were also present. The enhancement of muscle growth is not a generalized property of neural tissue. Implants of forebrain and somites yielded the small numbers of muscle strands found in cases of somites alone or somites plus notochord. That this action of the embryonic spinal cord on muscle growth need not be mediated by motor nerves is indicated by implants of somites from stage 33 embryos. Sizeable muscle masses developed in this series though motor nerves were not present.

The relation of the cortex to the formation of a perivitelline space in the eggs of Fundulus heteroclitus. CHARLES W. HUVER.

Unfertilized Fundulus eggs were centrifuged in an effort to determine if the cortically located cytoplasm is necessary for the formation of a perivitelline space. An International clinical centrifuge was used. Temperatures ranged from 22° C. to 26° C. during the experiments. All eggs were in a medium of 0.95 *M* sucrose solution. The control group consisted of 41 unfertilized eggs which were neither centrifuged nor pricked.

Preliminary centrifugation experiments at 846 × g. and at 3,390 × g. for 10 minutes caused the cytoplasm to concentrate at the centrifugal end of the egg. Cytoplasm could be seen because of its grey or brown color when concentrated. In all 28 eggs centrifuged in these early experiments the blastodisc formed at the concentration of cytoplasm. Cortical alveoli were densely packed in the blastodisc and a few were scattered on the egg surface. The size of the blastodisc was inversely correlated with the number of alveoli left in the egg cortex. Therefore, cortical alveoli may be regarded as indicators for the presence of cortical cytoplasm.

When 40 eggs were centrifuged at 4,320 × g. for 5 minutes, the cortex appeared to have been moved inside the egg in 15 cases. Since no cortical alveoli were seen on the egg surface, it is inferred that little or no cortical cytoplasm was present. The former cortex was clearly visible as a grey area filled with alveoli. This internally displaced cortex rounded up to form a blastodisc surrounded by yolk. Seven of the 15 eggs with internal blastodiscs were pricked with a glass needle 25 μ in diameter. In none of these eggs was there any formation of a perivitelline space. While in the controls and in the 25 other experimentals which had some cytoplasm remaining in a cortical position, a perivitelline space was formed regardless of whether they were pricked or not. These results suggest that cortically located cytoplasm is necessary for the formation of a perivitelline space.

The occurrence of a crystalline style in the marine snail, Nassarius obsoletus.[1] CHARLES E. JENNER.

The crystalline style is a specialized digestive apparatus found in all bivalve mollusks, but in gastropods its occurrence is believed to be restricted. The present report of its occurrence in *Nassarius obsoletus* is of special interest since: (1) this snail belongs to the order Stenoglossa, a group in which the style is believed not to occur; (2) this snail is frequently described as carnivorous, but the style is said not to occur in carnivorous snails; (3) this snail passes large quantities of sand and other particles of similar size through its digestive tract by peristalsis, a procedure not followed by any other snail reported to have a crystalline style.

The characterization of this snail as primarily carnivorous or as essentially a scavenger on dead animals is clearly in error for the following reasons: (1) the concentration of snails frequently encountered must require a food source far greater than provided by available animal substance; (2) the gut is frequently filled with bottom materials, mud and sand; (3) the possession of a crystalline style is primarily an adaptation for the digestion of starch, a plant product.

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[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

Seasonal resorption of the copulatory organ in Nassarius trivittatus and Littorina littorea.[1] CHARLES E. JENNER.

In *Nassarius obsoletus* the termination of seasonal reproductive activity is marked by the resorption of the copulatory organ in males (Jenner and Chamberlain, 1955). This has been found to be true also for *Nassarius trivittatus* and *Littorina littorea*, the phenomenon for the latter having previously been recorded for European waters (Tattersall, 1920, and others). As judged by copulatory organ resorption, *Nassarius trivittatus* from the shallow water of Eel Pond, Woods Hole, terminated seasonal reproduction by early July 1956. By the same criterion, *Littorina littorea* from mid-tide level at Nobska Point, Woods Hole, showed evidence of reproductive decline by late July; approximately 25 per cent reduction in males had occurred by August 25.

Electron microscopic observations on changes in the cortical cytoplasm after fertilization of Fundulus eggs. NORMAN E. KEMP AND MARGARET D. ALLEN.

Eggs were fixed in buffered (pH 7.4) 1% osmic acid in artificial sea water at various times: (1) before fertilization; (2) during breakdown of cortical alveoli, a process which starts at the animal pole one-two min. after insemination and is completed at the vegetative pole about one min. later; (3) after breakdown of cortical alveoli was complete; and (4) after the blastodisc was well-formed. In order to eliminate most of the yolk, eggs were fixed lightly (three-five min.) in osmic acid, then transferred to 50% sea water for removal of the chorion and bisection of the egg with iridectomy scissors. Unfixed yolk was washed out with a pipette and the partially fixed half-shells of cytoplasm and adherent yolk returned to osmic acid for about 15 min. to complete osmication, following which they were processed for methacrylate embedding and thin sectioning. Unfertilized eggs have short microvilli scattered over the surface. Some cortical alveoli are covered only by a thin layer of cytoplasm; others are embedded more deeply, some even resting against the interior yolk mass. After fertilization, alveoli come to the surface, burst and liberate their contents into the perivitelline space. Many alveoli form crater-like depressions in the surface cytoplasm during the process of extrusion. Cytoplasm may heap up to form a prominent elevated rim around an alveolar crater. Sides and floor of a crater come to be part of the surface of the egg as it smooths out after the cortical reaction is completed. The surface of the blastodisc possesses long pseudopodial processes indicative of great activity of the cortical cytoplasm. By contrast, the thin yolk gel layer peripheral to the blastodisc has a perfectly smooth surface.

Dehydrogenase activity in developmental stages of Asterias as measured with tetrazolium salts. EVELYN KIVY-ROSENBERG AND BENJAMIN W. ZWEIFACH.

A quantitative estimate of endogenous dehydrogenous activity during developmental stages of Asterias was sought using tetrazolium salts as indicators. The toxicity of the tetrazoles made such studies not feasible. As a result, an attempt was made to investigate specific substrate-dependent dehydrogenases at given stages (uninseminated ova, inseminated ova, blastulae, gastrulae). A method similar to the frozen tissue-slice technique was employed in which most endogenous activity is eliminated by freezing, and circumvents the problem of toxicity.

Having been removed from deep freeze, samplings of chosen developmental stages were thawed at room temperature. These were incubated anaerobically at 37–38° C. in a medium containing sea water, DPN, nicotinamide and one of a series of substrates (which had been used previously in tissue work): succinate, alpha-glycerophosphate, glucose, glutamate, malate, lactate, beta-hydroxybutyrate. No cofactors were used with succinate. Two tetrazolium salts were employed: 2, 3, 5 triphenyltetrazolium chloride (TTC) and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Final concentration of tetrazolium in the medium was under 0.5%. The amount of formazan was determined colorimetrically by a spectrophotometer. The substrate-dependent dehydrogenase activity was expressed in terms of micrograms per cubic milliliter of ova (or embryos).

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[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, E-356(C4).

It is evident that particular substrate-dependent dehydrogenases are more metabolically active than others and that there is a quantitative variation during development. Although the absolute quantity of reduced tetrazolium varied somewhat from one batch of eggs (or embryos) to another, it appears when TTC was used as the acceptor, that malate dehydrogenase activity is highest, with beta-hydroxybutyrate and alpha-glycerophosphate next in order though definitely lower. Other substrates yielded negligible values or none at all. With INT, malate, alpha-glycerophosphate and beta-hydroxybutyrate were highly active. Other substrates gave positive reactions but of much lower intensity. Limited observations indicate that alpha-glycerophosphate, malate, and beta-hydroxybutyrate-dependent dehydrogenases become less active soon after insemination and then increase again at gastrulation.

The chemical nature of bound oxygen in hemerythrin and in hemocyanin.[1] IRVING M. KLOTZ AND THEMIS A. KLOTZ.

Since ferrous ion can be released from deoxygenated hemerythrin and ferric ion from oxyhemerythrin, it has been suggested that the bound oxygen in the oxygenated protein is in the form of peroxide ion. Several tests for peroxide ion have been carried out, therefore. The most delicate test for H_2O_2 is the formation of a yellow color with a solution of TiO_2 in dilute sulfuric acid. To five drops of Ti(IV) test reagent in a spot plate was added, therefore, some oxyhemerythrin crystals. An orange color formed immediately around the protein; on further mixing the color was diluted to a strong yellow. Corroboration of the presence of peroxide ion was also obtained with the benzidine test. A few drops of a 4% solution of benzidine in glacial acetic acid were placed in a spot plate. To this solution were added a few milligrams of horse-radish peroxidase and then some crystals of oxyhemerythrin. A blue color developed immediately in the vicinity of the hemerythrin. Concentrated solutions of crystalline oxyhemerythrin, as well as laked blood, also gave positive benzidine tests. In contrast deoxygenated hemerythrin did not give the blue color. Both tests thus show that peroxide ion is released from oxyhemerythrin in acidified solutions.

In hemocyanin valence changes of the copper on oxygenation suggest that the bound oxygen is in the form of perhydroxyl free-radical ion. This species is a reactive intermediate known to appear in the metal-catalyzed decomposition of peroxide. Oxyhemocyanin was added, therefore, without peroxidase, to a solution of benzidine in glacial acetic acid. At 0° C., a stable blue color, characteristic of oxidized benzidine, was obtained, as would be expected if perhydroxyl radical were released from hemocyanin.

Crystallization of Busycon hemocyanin.[1] IRVING M. KLOTZ, THEMIS A. KLOTZ AND GEORG H. CZERLINSKI.

Attempts to prepare crystalline hemocyanin from the blood of *Busycon canaliculatum* by standard procedures used for other species have not proved successful. It has been found now, however, that an adaptation of the heparin method described by S. Cohen (1942) for other macromolecules does produce crystals.

Blood drained from the tissues of the conch is filtered through glass wool and then centrifuged at 4° C. at 2000 r.p.m. in the International Refrigerated Centrifuge. The solution is then decanted and dialyzed against distilled water at 4° C. for two days, with frequent replacement of the water outside the dialysis bags. The hemocyanin is then separated from the blood by ultracentrifugation at 35,000 r.p.m. in the Spinco Model L. The colorless supernatant is discarded, the concentrated hemocyanin at the bottom of the tube is redissolved in distilled water and the ultracentrifugation repeated. After the third ultracentrifugation, the concentrated hemocyanin is removed and to it is added solid sodium heparin, or a 10% aqueous solution, to a final concentration of 3%. Turbidity appears immediately but the mixture is permitted to stand overnight. The solid is then separated by centrifugation at 1500 r.p.m. Examination under the microscope reveals crystals, perfect ones being in the shape of hexagons.

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[1] This investigation was supported in part by a research grant, RG-4134, from the National Institutes of Health, Public Health Service.

Even this crystalline hemocyanin, in aqueous solution, shows two widely separated components in the Spinco analytical ultracentrifuge Model E. It is likely that the faster moving one is a dimer or higher aggregate of the monomeric form.

Pathways of glucose-C^{14} utilization in eggs of Arbacia, Mactra, and Chaetopterus.

M. E. KRAHL, A. K. KELTCH, C. P. WALTERS AND G. H. A. CLOWES.

From experiments with glucose-1-C^{14} (G1), glucose-2-C^{14} (G2), and glucose-6-C^{14} (G6), the authors reported here (1955) that: (a) glucose is oxidized in Arbacia eggs principally via the TPN shunt, the ratio of $C^{14}O_2$ from G6 to that from G1 being 0.07 for unfertilized and 0.12 for fertilized eggs; the glycolytic pathway is more important in 24 hour-old swimming embryos where the ratio is 0.28; (b) C^{14} from glucose appears in nucleoprotein; (c) dinitrocresol (DNC) inhibits glucose oxidation by the shunt, tending to divert glucose-6-phosphate into the glycolytic pathway; DNC also inhibits glucose-C^{14} conversion to nucleoprotein. These findings have been extended for Arbacia and found to apply in general to eggs and embryos of Mactra and to embryos of Chaetopterus. Representative total c.p.m. collected after 2 hr. incubation of 80 mg. eggs at 20° in 4 ml. sea water containing glucose-C^{14} (0.0006 M; 600,000 c.p.m.) are as follows. For Arbacia, just fertilized, in DNA from G1, 200, from G6, 700; in RNA from G1, 200, from G6, 400. For 24 hr. embryos, in DNA, from G1, 20,000, from G6, 18,000; in RNA, from G1, 12,000, from G6, 12,000. For Mactra the total c.p.m. into respiratory CO_2 with 0 and 0.0001 M DNC present were, respectively: unfertilized, from G1, 200, and 300; G2, 100 and 500; G6, 25 and 200; just fertilized, G1, 300 and 400; G2, 200 and 1600; G6, 200 and 700; 24 hr. embryos, G1, 16,000 and 22,000; G2, 7000 and 13,000; G6, 4000 and 11,000. The total c.p.m. into nucleoprotein of 24 hr. embryos with 0 and 0.0001 M DNC were, respectively: from G1, 27,000 and 4900; G2, 33,000 and 5900, G6, 33,000 and 6600. For Chaetopterus the total c.p.m. into respiratory CO_2 with 0 and 0.0001 M DNC were, respectively: for 24 hr. embryos, from G1, 5500 and 5000; G2, 1500 and 6500; G6, 800 and 3600. The total c.p.m. into nucleoprotein of 24 hr. embryos with 0 and 0.0001 M DNC were, respectively: from G1, 13,000 and 1400; G2, 16,000 and 2100; G6, 18,000 and 2000.

Improved fixing and staining methods for cellular structures in Ilyanassa eggs.

F. E. LEHMANN.

1. *Fixation.* Previous studies had shown that in Amoeba fibrous structures in cytoplasm are not sufficiently preserved by osmic or strongly acidic fixatives. A suitable rapidly penetrating and partly dehydrating fixative was found in a mixture of acetone and formalin; good preservation of cytoplasmic fibers was shown by the electron microscope. For Ilyanassa two mixtures were tried. A mixture of 80 ml. of 20% formaldehyde and 16 ml. of acetone is sufficient to fix fibrillar structures in the cytoplasm (cytoplasmic reticulum and asters). A slightly acidified fixative (80 ml. of 20% formaldehyde, 16 ml. of acetone, and 0.5 ml. of glacial acetic acid) gave good preservation also of interphase nuclei, spindles with chromosomes, and asters. In contrast to this, OsO_4 fixation of Ilyanassa eggs destroys astral structures, which are visible in living eggs. In order to stabilize structures containing nucleic acids and to prevent shrinkage by increasing rigidity of cell parts, chromic acid was added to the fixative (2 ml. of 10% chromic acid for every 5 ml. of fixative) 20 minutes after eggs had been placed into the fixative. After 5–20 minutes of exposure to this combination, eggs were rinsed for 1–2 minutes in distilled water; they were then ready for staining and mounting.

2. *Staining* by bromphenol blue-sublimate (after Mazia, Brewer and Alfert) showed cell boundaries, cytoplasmic reticulum, spindles and astral fibers clearly. For staining of total mounts of Ilyanassa eggs the original mixture (0.05 mg. bromphenol blue, 5 g. sublimate and 50 ml. H_2O) was diluted 20 times. Eggs were stained 10–20 minutes, transferred to 0.5% acetic acid for 20 minutes, and then to tap water for 5–20 minutes. They were then passed through alcohol, cleared in xylene and mounted in Canada balsam. Besides the blue cytoplasmic

structures the yolk granules stained partly purple, violet and green. The shape of the egg is very little distorted as compared with the living stages.

Factors inhibiting metamorphosis in tadpoles of the tunicate Amaroecium constellatum. William F. Lynch.

Tadpoles were placed in sea water solutions of 0.001 *M* potassium cyanide, 0.001 *M* sodium azide, 1.5% urethane (all at a pH of 7.8–8.0) and in sea water acidified to pH values of 4.8, 5.2 and 5.7. The number undergoing metamorphosis was determined: (1) after the last control had begun metamorphosis (30–70 min.) in eight observations, (2) at 7 hours, and (3) at 21 hrs. in three experiments. (1) After the last larva in the controls had begun metamorphosis the inhibition was: urethane, 100%; azide, 98%; cyanide, 97%; acidified sea water (pH = 4.8–5.2), 94%. Inhibition was completely reversible on removal of the tadpoles to sea water. (2) By 7 hours none of the tadpoles in urethane or azide (other experiments) and only 2.8% of those in cyanide and 8.3% of those in acidified sea water (pH = 5.7) had begun metamorphosis. Inhibition was reversible. (3) In another set of experiments inhibition was 100% in urethane and in acidified sea water (pH = 5.2) at 21 hrs. Larvae removed from either of these solutions showed a tendency for persistent swimming. Those removed from urethane changed their axes from 45 to 90° by 24 hrs. Those removed from acidified sea water only partially metamorphosed and then died. Inhibition was incomplete at 21 hrs. in azide and in cyanide. Unmetamorphosed tadpoles removed at 21 hrs. from azide developed normally and those from cyanide at a somewhat retarded rate.

Tadpoles placed in sea water solutions of 0.01% 2,4-dinitrophenol and 1.5% thiourea (both at a pH of 7.8–8.0) began metamorphosis and tail resorption as quickly as the controls, but subsequent stages were inhibited. The larvae contracted, became ovoid and the tunic imbibed much sea water; none of them changed their axes nor elongated by 24 hrs. Inhibition was completely reversible when tadpoles were removed from dinitrophenol at six hrs. or from thiourea at 1.5 hrs. Tadpoles in 0.15% chloral hydrate (pH = 8.0) showed 17% inhibition when the last larva of the controls had begun metamorphosis but they changed their axes from 10 to 90° by 24 hours; subsequent development was somewhat retarded.

A North American record of Rhopalura sp. (Orthonectida: Mesozoa), a parasite of the nemertean Amphiporus ochraceus (Verrill). Norman A. Meinkoth.

On July 3, 1956, a specimen of the armed nemertean *Amphiporus ochraceus* (Verrill), taken from among growths of the compound ascidian *Amaroucium pellucidum* dredged off Nobska Point near Woods Hole, Mass., was found harboring a strange parasite. These minute, ciliated, cylindrical, annulated organisms were noted both within the body of the host and swimming beside it on the slide.

The parasite measured 126 μ by 18 μ. Its body consisted of an outer layer surrounding an inner core of large cells, 19 to 23 in number, disposed in a single row anteriorly and double posteriorly, extending the length of the organism. Constrictions of the outer layer divided the body into a series of annuli, all of which with certain exceptions contained numerous refringent granules. Three annuli constituting an anterior cone were followed by two prominent annuli, each delimited by deep furrows. Behind these followed a series of six large annuli, each succeeded by a smaller granule-free annulus. The remaining posterior part was rounded, and somewhat longer than wide. All annuli bore cilia. Those on the first annulus were held rather rigidly anteriad as a tuft, while those on the posterior part, longer than the other body cilia, did little vibrating and trailed behind. The remainder of the body cilia, of about equal length and distribution, beat actively. All individuals encountered were essentially identical in the above characteristics. To date, of 171 more *A. ochraceus* examined, none has been found harboring the parasite.

Consultation with the literature indicates that these animals are females of a species of the genus *Rhopalura,* order Orthonectida, phylum Mesozoa. The species is similar to if not identical with *R. metchnikovi* Caullery and Mesnil 1901 or *R. linei* (Giard) 1879. This is believed to be the first reported occurrence of an orthonectid from North America.

Studies on accelerator and retarding factors of one species on the developing ova of an unrelated form.[1] VALY MENKIN, GABRIEL MENKIN AND LOUISE MENKIN.

Recent observations have indicated the presence of two distinct factors in the aqueous homogenate of the ovaries of *Arbacia punctulata.* These two factors conceivably play a significant rôle in the regulation of cell division. One of them markedly accelerates the rate of cleavage of sea urchin ova; the other displays considerable retarding activity of cleavage development. Since from a single homogenate two opposed factors are obtained by methods previously described, it is conceivable that the rate of cleavage of the fertilized ova is a resultant of these two opposed effects.

In a new series, it has now been shown in 10 distinct experiments that the accelerator factor derived from the homogenate of sea urchin ovaries speeds up the cleavage of the ova of a mollusk, *Spisula solidissima.* The accelerator factor is obtained by centrifuging the aqueous homogenate at about 10,000 r.p.m. in a Servall angle centrifuge for about one hour. The supernatant is then dialyzed against distilled water in a refrigerator for several days. The diffusate contains the accelerating factor. Its activity on the ova of *Spisula solidissima* has yielded the following results: the average number of ova is about 150 per cent greater in the experimental than that of the controls in the two-blastomeric stage. In the succeeding segmentation the incidence averages 100 per cent in the experimental over that of the controls. In a series of 13 experiments with the retarding factor of Arbacia ovaries on Spisula ova, the average retarding effect on the two-cell stage is 60.9% and 38.7% in the succeeding cleavage stage. Thus the factors obtained from an echinoderm are equally effective on the developing eggs of a mollusk, indicating that basic substances are evidently involved in the mechanism.

Further studies on some factors concerned in the regulation of cell division.[1] VALY MENKIN, GABRIEL MENKIN AND WILLIAM ROGERS.

Since there is a definite suggestion that the accelerator factor seems to be a nucleotide, some studies were undertaken to determine the effect of a few derivatives of nucleic acid on the cleavage rate of Arbacia ova. The pyrimidine base uracil appears in preliminary experiments to cause an initial acceleration in the incidence of the first segmentation, the figures being 63% in the experimental and 26% in the controls. In the succeeding cleavage the experimentals reveal an incidence of 75% as against 58% in the controls. This base is to be studied further. Cytosine seems to be incapable of altering the cleavage rate. For the pyrimidine bases doses of 75 γ per ml. were employed. Adenosine triphosphate (ATP), 75 γ per ml. on *Arbacia punctulata* ova yielded, in a series of six experiments, the following results: either a retardation or an acceleration in the cleavage rate. In the first segmentation ATP induced an average retardation of 13.1%, whereas in the succeeding cleavages an accelerating effect was noted amounting to 84.4%. The effects with ATP are therefore of a different order than those obtained with the accelerator cleavage factor fractionated from sea urchin ovaries.

The effects of the accelerator and the retarding factors derived from Arbacia ovaries, on the sperms of the clam, *Spisula solidissima,* were studied. Sperms were exposed for varying intervals (33 minutes to 4 hours) to the two factors, and to sea water as control. Ova of Spisula were then added, and the incidence of cleavage subsequently determined. The results of 9 experiments indicated 44.5% cleavage in the controls; 34.7% in the dish containing the accelerator cleavage factor; and 9.8% in the one with the retarding factor. Prolonged exposure to the accelerator factor seems to injure the sperms. This detrimental effect is very pronounced with the retarding cleavage factor. However, addition of the retarding factor following fertilization induced likewise retarding cleavage effects, indicating also a probable effect on the eggs themselves.

[1] Aided by grants from the U. S. Public Health Service, Sigma Xi, and Dr. A. Wander, S.A., Berne, Switzerland.

Effect of electric current on the contraction of the chloroplasts of Spirogyra. W. J. V. Osterhout.

Experiments were made on *Spirogyra* cells which contained single spiral chloroplasts. These cells were placed in 0.001 M NaCl solution which brought about no visible change in the chloroplasts. When a small amount of direct or alternating current (less than 0.2 milliampere) was passed through the solution each spiral chloroplast in less than ten minutes became detached, straightened, and contracted until it formed a small rounded mass. This contraction continued even after the current was turned off. A cell whose chloroplast was not visibly affected by the current sometimes showed signs of contraction after the current was turned off. This was not reversible. If the direct current was reversed there was no additional effect.

In order to make certain that these effects were not due to the heat produced in the circuit the following was done. A drop of gelatin whose melting point was 35° C. was put in the path of the current along with *Spirogyra* cells. Since the gelatin did not melt the temperature was less than 35° C. If a drop of gelatin was placed in a stender dish containing some water at 35° C. it melted. Cells of *Spirogyra* exposed to a temperature at 40° C. for one half hour in a stender dish of water showed no signs of contraction.

When the normal cells of *Spirogyra* were centrifuged the spiral chloroplasts became detached, straightened, and contracted. If the cells were centrifuged until only slight shortening of the chloroplasts was evident and removed to a stender dish of water no further contraction occurred.

Retinal action potentials in the eye of the scallop. Floyd Ratliff.

The scallop, *Pecten irradians,* has approximately one hundred eyes, each of which contains two retinae. Hartline found that nerve fibers from the proximal retina of an excised eye respond to the onset and continuance of illumination, while those from the distal retina respond only to the cessation of illumination. In the present study retinal action potentials were measured by placing one electrode on the posterior pole of an eye and inserting another into the eye near the margin of the lens. Upon stimulation by light the anterior portion of the eye becomes negative with respect to the posterior pole. This retinal action potential passes quickly through a maximum (approximately one millivolt when the eye is fully dark-adapted) and then subsides toward the resting level. Upon cessation of illumination the potential drops more rapidly toward the resting level. An "off" component, such as that found in many vertebrate and invertebrate eyes which respond to the cessation of illumination, is lacking. The absence of an "off" component is not due to inactivity of the distal retina, however. Simultaneous observations of retinal potentials in the eye and action potentials in the optic nerve showed that, at intensities of illumination great enough to produce a measurable retinal response, there was always a vigorous "off" discharge in the optic nerve when the light was turned off.

The course of the recovery of maximal sensitivity of the eye in the dark, following prolonged exposure to light, was determined by measuring the amplitudes of retinal potentials produced by stimuli of fixed intensity and one second duration spaced five minutes apart. Recovery is complete within about forty minutes.

Strain differences in viability following conjugation within variety 9 of Tetrahymena pyriformis.[1] Charles Ray, Jr. and Alfred M. Elliott.

Per cent of viable clones following conjugation of strains of variety 9 of *T. pyriformis* was studied as part of a program concerning strain relationships and genetics of biochemical differences between strains. Strains of variety 9 are known only from collections made in Panama or Colombia. Seven strains (TC. 105, 147, 156, 160, 258, 267 and 84) representing all collection sites and all five mating types were mixed in all possible combinations. Tests for selfing and for intervarietal mating were negative. Two hundred-twenty pairs were isolated singly into proteose-peptone from conjugating mixtures. Length of refractory period, amount of pairing, per cent of pairs giving viable clones, and stage at which death occurred for inviable pairs, were recorded.

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[1] This investigation was supported in part by research grants from the National Science Foundation and from the National Institutes of Health (PHS G3588).

All combinations of opposite mating types showed pairing. The refractory period of most combinations was about thirty-six hours (25° C.), although one paired at 24 hours and three at 60 hours. Per cent of pairing in various mixtures was poor to good. No relation was observed between length of refractory period, amount of pairing, and per cent of exconjugant clones. Of all crosses made, about 70% of the pairs died before fission of exconjugants, about 20% died after several fissions, and about 10% gave viable clones. Yield of viable exconjugant clones varied for different strains: *e.g.,* crosses involving TC267 gave 2% viable exconjugants; those with TC160 gave 20%. Three mating type IV strains gave 20, 13 and 2 per cent viable exconjugants. TC156 (III) and TC160 (IV) were isolated from the same original collection; they produced from all crosses 5% and 20% viable exconjugants, respectively, but when crossed with each other they gave no viable exconjugants.

Observation of cytological events associated with this widespread lethality and behavior of viable clones with continued cross and inbreeding is in progress.

Carbohydrates metabolized by cestode parasites of dogfish.[1] CLARK P. READ.

Calliobothrium verticillatum (Tetraphyllidea) and *Lacistorhynchus tenuis* (Trypanorhyncha) were removed from naturally-infected *Mustelus canis.* The worms were washed for 2 hours in several changes of filtered 40% sea water at room temperature (20–22° C.). Groups of ten worms (70 to 90 mg. of wet tissue) were transferred to Warburg flasks containing 40% sea water-bicarbonate (pH 7.2) and equilibrated for 15 minutes under 95% N_2–5% CO_2 in the 20° C. bath. Flasks were incubated for 60 minutes to determine the endogenous rate of acid production. Substrates were than added to make a final concentration of 0.01 *M* and the flasks incubated for an additional 60 minutes. Throughout each experiment readings were made at 20-minute intervals. Addition of acid to initial control flasks and to the experimental flasks showed that anaerobic metabolic gas is not produced by either of these cestode species. As indicated by an increase in acid production glucose and galactose are utilized by both tapeworms. Fructose, mannose, xylose, maltose, trehalose, sucrose, lactose, and raffinose are not metabolized. Non-utilization of the latter substrates was confirmed by analyses of the media before and after a three-hour incubation, using Roe's anthrone procedure for the sugar determinations. The rate of utilization of glucose and galactose is independent of the concentration in the range 0.001 to 0.02 *M.*

The extremely limited spectrum of carbohydrates metabolized by *Calliobothrium* and *Lacistorhynchus* resembles that of the cyclophyllidean cestodes, *Hymenolepis diminuta, Oochoristica symmetrica, Raillietina cesticillus,* and *Moniezia expansa.* Additional experiments are in progress to determine whether the dogfish cestodes resemble the cyclophyllideans in requiring the inclusion of carbohydrate in the host diet for normal growth and reproduction.

Recovery from x-irradiation effects at the cellular level. ROBERTS RUGH AND JOAN WOLFF.

It is more difficult to conceive of repair of structural damage consequent to x-irradiation than to a re-synthesis by the living system of molecules rendered unusable. Recovery of function at the cellular level is conceivable to a degree inversely related to time.

Henshaw originally described the recovery of Arbacia gametes following x-irradiation. The present experiments extend his work to determine the degree of recovery following different levels of exposure and different time intervals between x-irradiation and the fertilization of the Arbacia egg.

Eggs of Arbacia were washed and concentrated into a 40-cc. volume in filtered sea water, placed in a covered plastic dish, and x-irradiated at 2160 r/min. to from 36,000 r to 86,000 r. At intervals up to three hours after irradiation eggs were fertilized and the degree of cleavage determined at 1.5 and 2.5 hours after fertilization. To do this, eggs were fixed in 10% formalin with a trace of acetic acid, in sea water. Counts were made of 200 eggs from each sample.

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[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Johns Hopkins University, NR 119–353.

Cleavage was delayed in all eggs x-irradiated, the greater the exposure the greater the delay. However, eggs which were irradiated but not fertilized for one, two or three hours showed increasing recovery value (cleavage percentage) with increasing time. At the higher levels of exposure, which allowed no eggs to cleave, some eggs developed when the time between irradiation and fertilization was extended.

The above facts indicate that the type of damage inflicted upon the single-celled Arbacia egg by x-irradiation is reparable to a degree.

The effect of substrate on the length of planktonic existence in Nassarius obsoleta. RUDOLF S. SCHELTEMA.[1]

Veliger larvae of the mud snail *Nassarius obsoleta* (Say) were successfully reared through metamorphosis. In culture the larvae grew rapidly in 15-liter crocks with *Nitzschia closterium* (200,000/ml.) as a source of food. The length of pelagic existence extended from 20 to approximately 30 days. Planktonic life ended with the loss of the velum. Contrary to the accounts generally given for gastropod larvae, the velum of *Nassarius obsoleta* was cast off rather than resorbed. Shell height of the larvae at the time of setting varied between 0.5 and 1.1 mm.; the average was between 0.7 to 0.8 mm. Within a few days after metamorphosis, the shell darkened and become opaque. In a series of experiments, 40 cultures, each containing 20 Nassarius veligers, were maintained through metamorphosis in the laboratory. A substrate of sand and organic material ($< 140\,\mu$, sieved from natural bottom sediment of Barnstable Harbor) was added to one-half of the cultures. To the remainder of the cultures no substrate was added. In cultures of 20-day old larvae, 17% of the organisms with a substrate metamorphosed within 24 hours, while in those without substrate only 3% set during this period. Similarly in cultures containing 31-day old larvae, 90% of the larvae with a natural substrate metamorphosed, while only 19% of the larvae without substrate completed metamorphosis. Considering the average of all cultures between 20 and 31 days, in those with substrate added, 76% of the veligers set within 24 hours while in cultures without substrate only 24% completed metamorphosis within this time. These laboratory observations have considerable ecological significance since they may help to explain the distribution of newly metamorphosed Nassarius in the natural environment.

Electrophoretic separation of chromatophorotropic principles of the fiddler crab, Uca. G. C. STEPHENS, F. FRIEDL AND B. GUTTMAN.

The following observations were carried out in order to attempt to isolate and characterize the chromatophorotropic principles present in the sinus gland of the fiddler crab, *Uca pugilator*. Sinus glands were dissected and isolated in sea water as rapidly as possible until twelve glands were obtained. These were then placed on a strip of filter paper one-half inch wide and eighteen inches long which had been moistened with $M/15$ phosphate buffer at pH 7.0. The glands were then crushed and exposed to 450 volts DC at 5 to 8 ma. for 12 to 18 hours. On a separate strip in the same apparatus a sample of serum albumen was run to serve as a marker.

The results of this procedure were observed as follows. The strip of filter paper on which the glands had been placed was divided into ten equal units, five on each side of the point at which the glands had been crushed. Each unit was extracted with 0.5 cc. of sea water and 0.05 cc. of the extract was injected into each of five male fiddler crabs whose eyestalks had been removed at least twelve hours before use. The stages of the black, white, red, and yellow chromatophores of these assay animals were estimated before injection and observed again 20, 40, 60, and 120 minutes after injection of the test extract. A quite comparable assay procedure was used to study chromatophore concentrating activity of regions of the strip. In this case, the chromatophores (black, red, and yellow) of the assay animals to be used were dispersed by injection of a sinus gland extract one to two hours before injection of the filter paper extract.

By this procedure we were able to distinguish three distinct peaks of black dispersing activity, one dubious area of black concentrating activity, and at least one peak of dispersing activity and one peak of concentrating activity for the red and yellow chromatophores. At least two peaks of white concentrating activity were discernible. Each of these peaks mentioned can be stated to be distinct from any of the others by comparing the variation obtained in our several observations.

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[1] This work was done with the assistance of a summer fellowship from the Woods Hole Oceanographic Institution.

The rate of disappearance of the melanophore-dispersing hormone from the blood of the fiddler crab, Uca. G. C. STEPHENS, A. STRICKHOLM AND F. FRIEDL.

The rate of disappearance of the melanophore hormone in *Uca pugilator* was studied by the following technique. Assay animals were prepared by removing the eyestalks of male fiddler crabs at least twelve hours before they were to be used. Within this time the melanophores had become punctate so that the dispersing activity of an extract could be readily ascertained by observing the response induced on injection of the material concerned. The melanophore-dispersing hormone to be assayed was prepared by dissecting sinus glands from normal donor animals and grinding them to prepare a sea water extract.

An initial group of twenty-five to thirty animals was injected with 0.05 cc. of sea water containing the extract from one-half a sinus gland. One group of five of these animals was followed to ascertain the effect of this full strength injection. The remaining animals were divided into groups of five each. At suitable intervals after the initial injection (5 minutes, 15 minutes, 30 minutes, 60 minutes) 0.05 cc. of blood was withdrawn from each member of a group and injected into another group of five assay animals. The dispersing effect of the blood could then be followed in these secondary assay animals. As a control, blood from uninjected assay animals was withdrawn and injected into a second group of assay animals; no dispersion was obtained.

These experiments indicated that the dispersing hormone was present in the circulating blood of destalked assay animals in discernible amounts for approximately three hours after injection. In order to get a quantitative estimate of the amount present, readings of each assay group were made at fifteen-minute intervals after injection. The first seven estimates of melanophore dispersion were summed to give a measure of the effect of the extract or the blood which the animals had received. This information has been supplemented by a dilution curve measuring the effect of various concentrations of sinus gland extract, and by a measurement of typical blood volumes of the crabs (average 26.4% of body weight). This additional information permits calculation of the circulating hormone in sinus gland units.

Studies on activation in eggs of Urechis caupo, Nereis limbata and Asterias forbesi. HOWARD M. TEMIN.[1]

Activation in eggs of *Urechis caupo, Nereis limbata* and *Asterias forbesi* involves a series of linked depolymerizations of preformed layers or membranes: breaking of secondary valence and salt bonds in the cortex and disulfide bonds in the germinal vesicle. The process was studied by use of various reagents of known chemical action.

In eggs of Urechis the vitelline-fertilization membrane is an outer protein layer, soluble in non-electrolytes, and an inner calcium-proteinate layer. Activation involves breaking of secondary valence bonds in the egg cortex with a decrease in surface area; breaking of secondary electrostatic and then salt bonds in the sub-vitelline membrane layer causing membrane elevation and release of a compound which breaks disulfide bonds in the germinal vesicle.

In eggs of Nereis the vitelline-fertilization membrane is an outer layer soluble in alkaline thioglycolate and an inner layer soluble in citrate. The un-ionized jelly is secondarily bonded to the cortical gel. In solutions containing only monovalent ions (NaCl) or of agents which disrupt the cortex (sea water, pH 2.5; urea; sodium lauryl sulfate) the jelly is set free and the egg loses its depression. At a pH less than 9 the jelly passes through the outer membrane and then releases protons. At a pH of 10.5 the jelly does not pass through the membrane (Costello). Precipitation of the jelly to give a viscous mass depends on complexing with divalent ions. The germinal vesicle is soluble in alkaline thioglycolate, but not in urea, citrate, alkaline NaCl or solutions of ions.

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[1] National Science Foundation Pre-doctoral Fellow.

In eggs of Asterias the easily separable vitelline membrane is soluble in alkaline thioglycolate. On activation granules are released by breaking first secondary valence and then hydrogen bonds. These granules in the presence of ions precipitate on the vitelline membrane to give the fertilization membrane, which is also soluble in alkaline thioglycolate. The fertilization membrane expands in a divalent ion-free medium.

Spermatozoa in the oviducal gland of the smooth dogfish, Mustelus canis. LOIS E. TEWINKEL.

Fertilization of the elasmobranch egg, known to be internal, was thought to occur in the oviduct anterior to the oviducal gland until Metten reported (1939) that in *Scyliorhinus canicula* the oviducal gland, itself, serves as a seminal receptacle. Metten demonstrates the presence of spermatozoa exclusively within the shell-secreting tubules of the gland and considers that sperm are swept out, together with shell material, as the egg passes through this region.

Oviducal glands of *Mustelus canis* also contain sperm. Six mature females collected between June 28 and July 11 fall into the following three groups: a) two were post-partum and pre-ovulatory; b) one had ovulated two eggs; c) three had recently completed ovulation. Living sperm were found in washings of the oviducal gland in all specimens, but were not present in washings of more anterior or more posterior portions of the oviduct. Longitudinal sections of glands from each of the three groups of females show spermatozoa singly, in small groups, or in dense clusters, in the mouths or deep within the lumina of tubules, not only of the shell-secreting type, but also in more caudal "mucous" tubules. Occasional clusters or single sperm lie near the lamellar lining of the oviducal gland, but no sperm have been seen in albumen-secreting tubules or in more anterior mucous tubules.

Unlike the oviparous *Scyliorhinus*, which breeds throughout the year, the viviparous *Mustelus* presumably mates only between the birth of pups (late April to early June) and the onset of ovulation (June to early July). One would expect, therefore, to find few or no sperm in oviducal glands after gestation is advanced. In the glands studied, even after ovulation has ceased, sperm in large numbers are present, especially in the caudal "mucous" tubules, but whether this is the case in later pregnancy cannot be answered until glands from such females are examined.

Acetylcholine and frog brain oxygen consumption. ELBERT TOKAY.[1]

Since there seems relatively little known about the influence of acetylcholine (ACh) on brain metabolism, a study of the effect of the drug on frog brain oxygen consumption (standard Warburg technique) was undertaken for the purpose of correlating the findings with electroencephalographic results.

Optic lobes and cerebral hemispheres were separated (razor blade slicing) from freshly isolated frog (*Rana pipiens*) brains. Each Warburg flask contained four optic lobes or cerebral hemispheres (from different brains) in Ringer's. Flasks were gassed with pure oxygen and equilibrated at $30° \pm 0.5°$ C. At 10-minute intervals, readings of "normal" oxygen consumption were taken for one hour and then for two additional hours after tipping in side-arm contents (acetylcholine chloride Merck or Ringer solutions). Each ACh flask was duplicated by a Ringer flask, both containing similar parts of the same brains. Oxygen consumption ($mm.^3$ per g. of wet tissue weight) was calculated and the Q_{O_2} ($mm.^3$ O_2 consumed per g. per hour) derived from the slope of the plotted curve.

The "normal" Q_{O_2}'s are in agreement with those of previous workers. ACh was used in concentrations ranging from 10^{-3} to 10^{-9} g. per ml., in steps of tenfold dilution. Current results indicate that high concentrations of ACh depress oxygen consumption, 10^{-3} markedly and 10^{-4} somewhat less markedly. Lower concentrations (10^{-5} and below) tend to increase oxygen consumption more often than decrease it. More definitive conclusions and any differential effects on parts of the brain must await further experimentation and statistical evaluation.

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[1] This investigation was supported in part by research grant B-918 from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service.

A comparison of two inhibiting agents in Tubularia. KENYON S. TWEEDELL.

Regeneration in Tubularia can be inhibited with either a culture medium filtrate or with adult tissue extracts. Suspected differences in the active components were investigated.

An effective concentration of inhibitor water is dependent upon the tissue source and the concentration. Pieces of stems only showed no inhibition while amputated hydranths gave initial inhibition at a concentration of 125. Stems plus intact hydranths were the best source; the minimum effective number necessary is about 200 to 250 per 200 ml. collected for 18 to 24 hours. During this time an average drop in pH of the collecting medium of 0.5 was noted. The inhibitor strength is reduced when it is collected in boiled sea water. Refrigeration for 24 hours at 7° C. or boiling will destroy it. Adsorption with Norite A or synthetic resins will completely remove its activity but centrifugation at 21,000 G has no effect. The fresh filtrate gives a positive test with ninhydrin and the Feulgen-Schiff reagents. The regenerating stems are most susceptible to inhibition during the first 21 hours (proximal ridge stage). Beyond this, little effect is noted. Aeration of the regenerates during the first 20 hours improves regeneration in inhibitor water.

The supernatant from tissue breis of mature hydranths was collected by centrifugation (21,000 G). The minimum inhibitory dose was between 20 and 25 hydranth equivalents wherein stems required longer to regenerate (96 hours) and were reduced in size. Complete inhibition occurred between hydranth equivalents of 35 to 50 (a concentration of $\frac{1}{15}$ to $\frac{1}{10}$ in the culture medium). The tissue extract is highly resistant to sterilization, centrifugation and can be refrigerated indefinitely. The supernatant gives positive protein tests but freshly collected or boiled extract will readily dialyze and completely inhibit regeneration. Dialysis of the extract against running sea water for 24 hours with subsequent application to regeneration stems show a total loss of activity.

Attempts to breed an x-ray resistant clone of Paramecium. RALPH WICHTERMAN.[1]

In an attempt to breed radio-resistant paramecia by selection and cultivation of survivors of x-radiation, clonal cultures of *Paramecium multimicronucleatum* were irradiated repeatedly at intervals extending over a period of 13 months. For irradiation, 200 specimens were placed in each of 4 Nylon syringes (2 cc.) and, in one irradiation operation, given different dosages by the removal of a syringe at intervals from the x-ray generator. Most of the paramecia were irradiated with 100 to 250 kr in steps of 50 kr but occasionally higher dosages were used. Immediately after all irradiation exposures, survivors of the different dosages were placed in test-tubes of lettuce medium containing *Aerobacter aerogenes* as the food source. Upon regaining reproductive ability, progeny of survivors were then harvested, placed again in the syringes and irradiated as before. In some cases the next dosage was increased. As an example a clonal sample which received an earlier dosage of 100 kr later received 150 kr. After 13 successive and varied irradiation exposures of 4 sets of syringes, the clonal cultures of paramecia have received in this manner a cumulative dosage amounting to 1800 kr. Specimens are presently reproducing although at a slower rate of division than the unirradiated controls even after one year following the last irradiation. In addition to the reduced fission rate, the heavily irradiated clones after one year contain specimens which reveal the following: reduced swimming activity and altered behavior, reduced size, altered body shape and complete loss of all micronuclei. Unirradiated controls have three micronuclei.

Instead of being stream-line as in the controls, the paramecia from cumulatively irradiated clones are more ellipsoidal. Fission rate of well-fed controls occasionally reaches three divisions per day, generally not less than two. The successively irradiated clones that have received 1800 kr contain specimens which rarely reach two divisions per day even when last x-rayed one year earlier.

Using the same dosages, results indicate that specimens from the successively irradiated clones are more radio-sensitive than those irradiated for the first time.

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[1] Part of a project aided by a contract between the Office of Naval Research, Department of the Navy, and Temple University (NR 135-263) and the Committee on Research, Temple University.

The micromanipulation of Arbacia eggs. F. J. WIERCINSKI.

Preliminary to a series of experiments with *Arbacia punctulata* egg cells it was necessary to develop a suitable microinjection technique. Egg and sperm suspensions were obtained from Arbacia by the electric method of stimulation. Fertilized and unfertilized eggs were pricked with a fine micropipette in a thin film of sea water suspended over a moist chamber. The micropipette was mounted in the upward position. With this technique many of the fertilized eggs did not develop to the two-cell stage because of injury from high surface tension phenomenon along the edge of the hanging droplet. A depression slide marked into two-millimeter squares containing 150 λ of sea water for 50 to 80 eggs was found to be adequate for normal development. After ten to fifteen minutes following fertilization, eggs were successfully microinjected with approximately 3 cubic micra per egg of 0.5% gelatin solution and showed normal development. The micropipette with a fine shaflet and a one-micron opening at the tip was mounted downward so that the injection was made into the cell as it rested on the surface of the slide. Dark field illumination with a magnification of 100 × was used to advantage to observe the position of the tip of the micropipette. The location of the eggs for control and experiment was noted by the squares on the slide. In late June, 1956, the fertilization percentage was 80. The jelly coat did not cling to the micropipette. In the middle of July, the jelly coat of the fertilized eggs was very tacky and the cells clung to the micropipette. The fertilization percentage was 95.

The physiology of the heart in marine fish. CHARLES G. WILBER.

For several years a variety of physiological studies have been made on the heart of marine fish available in the Woods Hole area. There have been suggestions in the literature that cardiac rate in fish may vary with size as in mammals. In order to test this, data from numerous species have been assembled. There is strong evidence that at a given temperature large fish have appreciably slower hearts than do smaller. For example here are a few average values in beats per minute for fish arranged in order of decreasing size: Roccus, 20; Opsanus, 40: Prionotus, 50; Tautogolabrus, 60; Fundulus, 100. These are values taken at an ambient temperature of 22° C. The toadfish, Opsanus, has a heart which is very refractory to many drugs: fairly large doses of decamethonium, atropine, and darstine cause no changes in the electrocardiogram of this species. Massive doses of darstine (80 mg. intravenously) cause an A-V dissociation and an eventual doubling of conduction time from pacemaker to ventricle. The latter chamber seems to be more sensitive to the drug than is the pacemaker. Decamethonium in the tautog results in impure auricular flutter; conduction time thru the ventricular muscle is unchanged but there is a significant prolongation of the refractory period. These studies are continuing.

A rapid method for recognition of specimens of Littorina littorea infected with trematode larvae. CHARLES H. WILLEY.

In response to the need for snails infected with *Cryptocotyle lingua* for experimental purposes an attempt was made to discover some external feature of the snail which would indicate that it was infected. The isolation method with examination of the water for emerged cercariae is too time-consuming and the snails show a high mortality rate following long isolation. Some snails even though infected will not shed cercariae during isolation for several days and the isolation method fails to identify immature stages of infection.

Observation indicated that the foot of an infected snail (*Littorina littorea*) becomes a dark yellow to brown in color in contrast to the whitish foot of uninfected specimens. To detect infected individuals, collections of snails are placed in sea water in tall glass containers such as battery jars and allowed to crawl up the sides. On the basis of the color of the foot seen from outside the glass, specimens can readily be sorted into infected and uninfected categories. In five collections of snails, the results have consistently been positive, checked by crushing and examining all the snails, both brown- and white-footed. Unless the specimens had a brownish foot, they were not infected. In one experiment 82 snails were assorted into two groups, 60 white foot and 22 with brown foot. Crushing and examination showed no infections among those with white foot and 21 of the brown-footed specimens were infected. Similar results were obtained with other collections. Most of the infected snails harbored *Cryptocotyle lingua* but two other species were encountered in the infected group. Some were in early stages of infestation which would not have been detected by isolation techniques.

Induction of premature cleavage furrows in the eggs of Arbacia punctulata. ARTHUR M. ZIMMERMAN [1] AND DOUGLAS MARSLAND.[2]

These experiments indicate that the furrowing reaction, which normally is not scheduled to occur until telophase, can be induced to occur much earlier, starting, in fact, at about the 12th minute (at 20° C.) following insemination.

The induction treatment consists of pressure-centrifuging the fertilized eggs at high (8000–12,000 lbs./in.^{2}) pressure and at high (41,000 × G) force, for periods ranging up to 5 minutes. The temperature in all the experiments was kept constant at 20 ± 0.3° C.

The premature furrows appear 2–4 minutes subsequent to centrifugation, always at right angles to the centrifugal axis. Usually the furrows impinge from the equator of the cell, although sometimes they are displaced toward the centripetal end. Frequently they cut completely through the cell and do not recede. However, premature furrows, induced not more than 10 minutes prior to the normal time of furrowing, usually recede as soon as the normal furrows appear; and the normal furrows almost always come in at right angles to the premature ones. The eggs do not appear to be damaged appreciably, since the treated specimens gave rise to apparently normal plutei.

The greatest frequency of premature furrowing, which in many experiments involved virtually 100% of the eggs, was observed at 30–35 minutes after insemination. At 12,000 lbs./in.^{2}, a maximum frequency was obtained with 300 seconds of centrifugation, and there was a gradual decline in frequency with lesser durations of treatment. At lower pressures (8000 and 10,000 lbs./in.^{2}) the centrifugation times were longer.

Preliminary observations indicate that the induction of premature furrowing may be related to the rupturing of the nuclear membrane. No intact nuclei can be seen, either by phase or ordinary microscopy, in the pressure-centrifuged eggs, whereas nuclei can be seen in companion eggs centrifuged at the same force but not under pressure. Moreover, pressure-centrifuged premature furrowing eggs, stained by Feulgen (or the acetocarmine) technique, do not show intact nuclei, whereas the control cells do. The experimental eggs show just a small clump (sometimes two clumps) of densely packed Feulgen-positive material, lying in the vicinity of the furrow and displaying a diameter about ⅓ that of an intact nucleus. It is suggested, therefore, that the furrowing may be induced by a substance or substances released by the breaking of the nuclear membrane.

Pressure-centrifuge studies on mast cells. ARTHUR M. ZIMMERMAN,[1] JACQUES PADAWER [3] AND DOUGLAS MARSLAND.[2]

The pressure-centrifuge has been used extensively for studying sol-gel equilibria in Amoeba, Elodea and various marine eggs, but not in somatic mammalian cells. In this study, mast cells from rat peritoneal fluid were centrifuged under varying hydrostatic pressures at 41,000 × G and 20° C. It was found that the relative gel strength of these cells (expressed as the logarithm of the centrifugation time required to effect a distinct deformation in 25% of the cell population) is inversely proportional to the pressure. This relationship is similar to that found in all other cellular types that have been studied. Control mast cells, centrifuged at 41,000 × G at atmospheric pressure for periods up to 8 minutes (the longest centrifugation used in this study), were not measurably deformed. The deformation effected by the centrifugation was found to be spontaneously reversible at room temperature within approximately 30 minutes following return to atmospheric pressure.

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[1] Fellow of the Lalor Foundation, 1956.

[2] Work supported by the National Cancer Institute, Grant C-807 (cont.)

[3] Post-doctoral Research Fellow, American Heart Association. Supported in part by grants from the Damon Runyon Memorial Fund for Cancer Research (DRG 360) and from the American Heart Association.

Preliminary experiments show that mast cells obtained from 22-week-old rats may be appreciably more gelated than cells similarly obtained from 5-week-old animals. This result suggests that the aberrant morphology of mast cells encountered in old rats may be related to a progressively increasing gelational state of these cells during the aging process of the animal. Other cellular types of the peritoneal fluid (macrophagic elements and eosinophils) were not studied quantitatively. However, these cells were also deformed by the pressure-centrifugation. In fact, they seem to display a weaker gel structure than the mast elements.

LALOR FELLOWSHIP REPORTS

Sensory and motor relationships of a crustacean central ganglion. MELVIN J. COHEN.

Input-output relationships of the supraoesophageal ganglion in the lobster *Homarus americanus* were studied by stimulating statocyst afferents and recording the response evoked in occulomotor nerve fibers leaving the ganglion. Circulation to the exposed ganglion must be intact and the exposed portion of the central nervous system bathed in a balanced physiological solution in order for transmission through the ganglion to occur.

Movement of the statocyst sensory hairs evoked a response in occulomotor fibers which usually did not participate in the "spontaneous" activity of this nerve. Cutting both circumoesophageal connectives caused a burst of activity in the motor nerve followed by a gradual decrease in the number of spontaneously active units until only 1–3 fibers remained firing 15 minutes after cutting the connectives. The spontaneous activity in these remaining units was very rhythmic in contrast to the irregular spacing of impulses in the statocyst afferent neurons. Some occulomotor fibers increased in frequency up to 40/sec. when ipsilateral statocyst hairs were moved laterally toward the vertical, and decreased to 1–2 impulses/sec. when the same hairs were moved medially toward the horizontal. Here the frequency changes in the motor fibers seem to parallel those of the sensory neurons. Other occulomotor fibers increased in frequency only when statocyst hairs were moved medially toward the horizontal. This direction of hair movement is usually associated with a decrease in the frequency of firing in statocyst neurons. It appears, therefore, that a decrease in the level of spontaneous firing in certain statocyst afferents can serve as an adequate signal to the central nervous system and evoke a rise in frequency in specific fibers of the occulomotor nerve.

Invertebrate metabolism in vitro not affected by estradiol. DWAIN D. HAGERMAN.

Estradiol-17β stimulates the oxidative metabolism of human endometrium and placenta *in vitro*. The stimulation can be demonstrated in tissue slices or cell-free soluble enzyme preparations, and is the result of a specific activation of a DPN-linked isocitric dehydrogenase. In a search for metabolic effects of estrogens in other species, a variety of invertebrate tissues were incubated in Warburg vessels in the presence or absence of estradiol (4×10^{-6} moles per liter). The incubation medium was filtered sea water, to which was added glucose (11.1 millimoles per liter) or potassium pyruvate (10 millimoles per liter) in some experiments. The gas phase was air. Tissues were incubated at 24–25° C. for four hours. Oxygen consumption was measured manometrically and conventional chemical techniques were used for the analysis of glucose, pyruvic acid, glycogen, and lactic acid.

No effect of estradiol on the rates of oxygen consumption, glycogen utilization, or lactate production was found in the ovaries and contained eggs of *Arbacia punctulata, Asterias forbesi, Mactra solidissima, Venus mercenaria, Busycon canaliculatum, Carcinides maenas, Homarus americanus,* or *Limulus polyphemus.* No effect of estradiol on glucose utilization or pyruvate utilization was found in the ovaries and contained eggs of *Arbacia punctulata, Asterias forbesi, Mactra solidissima,* or *Busycon canaliculatum.* Moreover, no effect of estradiol on these metabolic functions was found in the whole body of *Microciona prolifera,* the ctenidia or testes of

Loligo pealei, the gills or testes of *Callinectes sapidus,* the testes of *Homarus americanus,* or the liver of *Limulus polyphemus.*

Estrogens have been reported to be present in some of these tissues. Our metabolic experiments do not reveal any invertebrate estradiol-enzyme relationship similar to that found in man, and it is concluded that the estradiol-sensitive, DPN-linked isocitric dehydrogenase is not present in invertebrate tissues.

Methods for investigating the location of the photoperiodic receptors in insects. A. D. Lees.

Although it is well known that the induction of diapause in many insects and mites is controlled by the length of day, the site of absorption of the photoperiodic light energy has not yet been identified. In this connection the observation by Tanaka that the larvae of the oak silkworm *Antheraea pernyi* still respond to photoperiod after extirpation of the ocelli is of considerable interest. Two techniques which may prove of use in the identification of the receptors are being tested currently, using the larvae of *A. pernyi* as material. (I) Localized illumination can be achieved by the topical application of a transparent cellulose paint containing a blue-fluorescing substance, such as anthracene. The insects are then exposed for part of the photoperiod to a U.V. source with maximum emission at 365 $m\mu$. The feasibility of this method rests upon the fact that insects in general exhibit greatest sensitivity to the blue region of the spectrum. (II) Using low incident light intensities to minimize light scattering, it may be possible to "silhouette" the sensitive areas by covering them with an opaque black paint. Since all five larval instars of *A. pernyi* are light-sensitive, the treated areas must be re-covered after each moult.

Contractility of glycerinated Vorticellae. Laurence Levine.

The technique of glycerination was applied to various species of Vorticellae in an attempt to elucidate some features of intracellular environment necessary for coiling of the spasmoneme (condensed myonemes).

Three species of Vorticellae, *campanula, nebulifera* and *convallaria,* maintained on an egg yolk infusion were used. Each species was very sensitive to glycerol because they invariably coiled tightly when immersed at 0° C. even in concentrations as low as 0.05 per cent. However, if the glycerol was 4 *mM* in EDTA (pH 7) such coiling was prevented. The glycerination procedure adopted, therefore, was immersion of animals previously washed in de-ionized water in 20 or 50% 0° C. glycerol and 4 *mM* in EDTA. Vorticellae glycerinated according to this recipe for as long as one month showed excellent preservation of the spasmoneme and other cellular features.

Physiological integrity of the spasmoneme was also retained. The coiling so characteristic of the living spasmoneme was produced by the addition of $CaCl_2$ in concentrations as low as 0.3 *mM* in 0.5 *M* KCl. Coiling was reversed through application of 4 *mM* EDTA and preparations were cycled repeatedly before contractility was lost. Contractility could not be restored through application of ATP and ions known to contract glycerinated rabbit psoas. Divalent ions such as magnesium and manganese also produced coils but were not as effective as calcium.

The retention of contractility was dependent upon species, concentration of glycerol and extraction time. Stalks of *Vorticella convallaria* lost contractility after short extraction in 20% glycerol, whereas coiling ability was maintained for longer periods in 50%.

These observations suggest that an extractable calcium-activated factor is responsible for spasmoneme coiling.

Regulation of arginine biosynthesis in Escherichia coli. W. K. Maas.

Although *E. coli* cells are able to synthesize their amino acids from simple nitrogen and carbon sources such as ammonia and glucose, when an amino acid is supplied in the culture medium, the bacteria will utilize it in preference to synthesizing their own. In order to elucidate this regulatory mechanism, the effect of externally supplied arginine on its own biosynthesis was studied. Arginine was chosen because most of the enzymes involved in its biosynthetic pathway have been extracted and the reactions catalyzed by them characterized. It was found that arginine inhibits the synthesis of transcarbamylase, the enzyme which couples ornithine with carbamyl phosphate to form citrulline. In growing cultures, in the presence of 10 micrograms/ml. of arginine, no transcarbamylase is formed. After removal of arginine the enzyme is resynthesized rapidly. Citrulline, on the other hand, does not inhibit synthesis of transcarbamylase. Studies are in progress on the effect of arginine on other enzymes involved in arginine biosynthesis.

The transcarbamylase system also offers an opportunity for studying the conditions required for the synthesis of a constitutive enzyme. Experiments have been carried out to see whether or not the presence of ornithine is necessary for enzyme synthesis. For these studies, a mutant unable to synthesize ornithine was used. The cells were first grown on arginine to exhaust transcarbamylase and then transferred to either citrulline or ornithine. It has found that transcarbamylase was resynthesized as rapidly in the presence of citrulline as in the presence of ornithine. These results indicate that, in contrast to the synthesis of adaptive enzymes, here the substrate may not function as an inducer for enzyme formation.

The ATPase activity of frog myosin. G. W. de Villafranca.

In studying the ATPase activity of frog muscle it was found that methods normally used for preparation of myosin from rabbit muscle always resulted in actomyosin. Even centrifugation of purified actomyosin at 110,000 g in the presence of ATP failed to yield actin-free myosin. Myosin, however, was obtained by centrifuging twice precipitated actomyosin in 0.6 M KCl and 0.1 M $MgCl_2$ at 110,000 g for 30 minutes after the actomyosin had been previously cleared by centrifugation in 0.6 M KCl at that force for 20 minutes. The supernatant after Mg^{++} treatment was then treated with an equivalent amount of Versene to remove the ATPase inhibiting Mg^{++}. It was then precipitated twice by dilution with 10 volumes of cold, ion-free water and, between precipitations, dialyzed overnight against 3 changes of 0.6 M KCl. There was no change in viscosity of the myosin upon addition of ATP, it had an intrinsic viscosity approximately that of rabbit myosin, and it had pronounced ATPase activity (Q_P about 1200).

Three types of preparations (short extraction with Hasselbach-Schneider solution, 24-hour extraction with Weber-Edsall solution, and myosin prepared as described) showed the same ATPase characteristics and yielded Q_P-s ranging from 500–1400 at 24° C. The pH optimum was found to lie between pH 9 and 9.5 with occasional smaller peaks in the pH 6–7 region. Calcium activated strongly with an optimal concentration of 5×10^{-3} M while magnesium either inhibited or gave the same activity as the absence of divalent ions. Increasing the KCl concentration from 0.03 to 0.24 M progressively decreased activity. The enzyme split only 40–50% of the 10 min. P. Optimal activity was obtained at 24° C.; pre-incubation for 15 minutes resulted in slight inactivation (10%) at 37° C. and almost complete inactivation (94%) at 45° C.

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A NEW PHYCOERYTHRIN FROM PORPHYRA NAIADUM[1]

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Svedberg and Katsurai (1929) proposed a phylogenetic nomenclatural system for the classification of the phycobilin pigments of the algae. They designated the phycoerythrin and phycocyanin from the red algae as R-phycoerythrin and R-phycocyanin, respectively, and the corresponding pigments from the blue-green algae as C-phycoerythrin and C-phycocyanin. In general, these pigments exhibit the following absorption maxima:

Pigment	Approximate absorption maxima mμ			
R-phycoerythrin	495	540	560	
C-phycoerythrin		550		
R-phycocyanin		550		615
C-phycocyanin				615

This system has proved inadequate in several instances (Kylin, 1912; Lemberg, 1930; Kylin, 1940; Haxo, *et al.*, 1955) in that phycobilin pigments other than the above types, as judged from their absorption spectra, have been isolated. In spite of the apparent shortcomings of the Svedberg and Katsurai system of classification, no new system has been proposed.

A new phycoerythrin has now been isolated from *Porphyra naiadum* and it is proposed that this pigment be called B-phycoerythrin (tentatively so designated by Blinks, 1954). This differs from known phycoerythrins in having two absorption peaks, at 545 and 565 mμ. The isolation, purification and some properties of this pigment will be discussed.

SOURCE

Porphyra naiadum Anderson is a member of the most primitive red algal order, the Bangiales. There is now some question as to whether it belongs in the genus *Porphyra,* since its life cycle is different. This is currently under study by Prof. G. J. Hollenberg; pending his description of a new genus, we must use the current name. The thallus is one cell thick, extremely delicate, and yields its pigment readily into fresh water in a few hours. It is found growing only upon a marine flowering plant, *Phyllospadix.*

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MATERIALS AND METHODS

Masses of thalli were stripped from the host plant and washed with distilled water. The washed algal mass was then just covered with distilled water and kept at 5° C. for about 15 hours. The supernatant, which contains the water-soluble phycobilin pigments, was separated from the algae by centrifugation at 15,000 times gravity for one hour. By this procedure approximately 75 per cent of the total phycobilins present can be extracted. The supernatant was filtered twice through Whatman No. 1 filter paper and the filtrate centrifuged at 20,000 *g* for twenty minutes. The pH of the pigment solution at this stage was 6.8–7.0. The pigment solution was then dialyzed in "Visking" tubing for 12 hours at 1° C. against 0.1 *M* acetate buffer, pH 5.0. The dialyzed pH 5.0 pigment solution constituted the stock solution and will, in the future, be referred to as such.

RESULTS AND DISCUSSION

Purification and crystallization

The general method of purification and crystallization of phycobilin pigments, which has varied slightly from investigator to investigator, involved precipitation with ammonium sulfate (Kylin, 1912; Kitasato, 1925; Lemberg, 1928). This precipitation is carried out after the algae have been extracted for several days at room temperature under slightly basic conditions.

This procedure was attempted on freshly extracted stock pigment solution from *P. naiadum:* it consistently failed to crystallize phycoerythrin and phycocyanin although concentrated ammonium sulfate precipitated an amorphous mass. If the stock pigment solution was allowed to stand at room temperature for several days, then phycoerythrin (but not phycocyanin) could be crystallized by this method. Bannister (1954) working with the blue-green alga *Synechocystis* apparently encountered similar difficulties in trying to crystallize with a freshly extracted pigment solution. It seems very likely that the phycoerythrin obtained by the classical procedure may be a modified pigment.

In contrast to ammonium sulfate treatment a freshly prepared stock pigment solution yielded well-formed phycoerythrin crystals on simply standing, in the cold, for about 24 hours at pH 4.5 These crystals were separated by centrifugation, washed in acetate buffer at pH 4.5, and redissolved in water adjusted to pH 7.5. Recrystallization could be carried out by reacidification to pH 4.5. The first crystallization gave an estimated 20 per cent yield of the total phycoerythrin present, while the second and subsequent recrystallizations were quantitative. The reason for this low yield in the original crystallization will be discussed in a subsequent communication. (Suffice it to say here that the non-crystallizable fraction appears to have an iso-electric point (if at all) in very acid ranges.)

The absorption spectra of the original crystals and three-times recrystallized B-phycoerythrin are presented in Figure 1. The main absorption maximum is at 545 mμ; this value did not vary between pH 5.0 to 7.0. Phycoerythrin that is kept at pH 9.0 for 6 hours has the same absorption maximum but the blue (400–450 mμ) and red (600–700 mμ) absorption is increased by about 300 per cent. Recently Haxo *et al.* (1955) have found that the phycoerythrin from *Porphyridium* was modified above pH 7.3. Our findings are also consistent with theirs in that we find that phycocyanin from *P. naiadum* is irreversibly bleached at pH 9.0. In contrast to the phycoerythrin from *Porphyridium*, however, neither the native nor the crystalline B-phycoerythrin from *Porphyra naiadum* tended to form additional "shoulders" or absorption maxima under alkaline conditions.

In addition, it is apparent that the original B-phycoerythrin crystals exhibit a "shoulder" in the 565 mμ spectral region which is lacking in the three-times recrystallized preparation. Whether this absorption represents an impurity in the original crystallization or a modification of phycoerythrin on repeated recrystallization is unknown at the present time. There are, however, reasons for believing that the latter may be the case. On repeated recrystallization it was found that the B-phycoerythrin solubility at pH 7.5 decreased with the number of times the pigment was recrystallized. Also the stability of the native and crystalline phycoerythrin to hydrogen peroxide is different. Table I gives the reduction in optical density at 540 mμ of the two pigment solutions when treated with varying hydrogen peroxide concentrations. The pH of both solutions was 5.8.

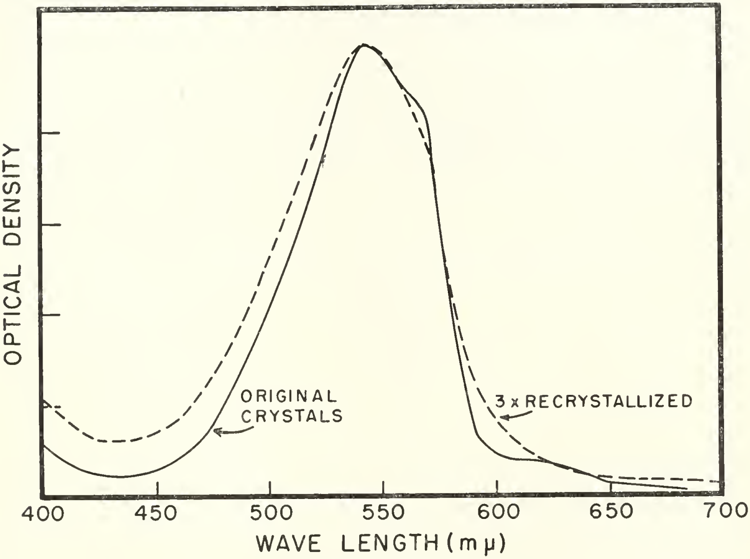

FIGURE 1. Absorption curves of once- and three-times recrystallized B-phycoerythrin. Solid line, original crystals; dashes, three-times recrystallized.

B-phycoerythrin was also isolated by the chromatographic method of Swingle and Tiselius (1951). Haxo *et al.* (1955) have successfully separated the phycobilin pigments, including allophycocyanin, from several algal species by this method. The latter pigment is also present in *P. naiadum* and this was the only method we found to isolate allophycocyanin and phycocyanin in relatively pure form.

The order of pigment elution from the column depends upon the pH of the elutant. Using 1 to 2 *M* acetate buffer at pH 5.0 as an elutant, the pigments come off the column in the following order: phycocyanin, B-phycoerythrin, a mixture of phycoerythrin and phycocyanin. Allophycocyanin could not be eluted from the column at pH 5.0; however, it was eluted with 0.1 *M* phosphate buffer, pH 7.0. When phosphate buffer (0.05 to 0.1 *M*), pH 7.0, was used the elution order of the pigment was: B-phycoerythrin, a mixture of phycoerythrin, phycocyanin and allophycocyanin, and finally allophycocyanin.

The phycoerythrin isolated by this method was, however, never completely free of phycocyanin and repeated chromatography did not remove this impurity.

Ultracentrifugation

Svedberg and his collaborators determined the molecular weight of crystalline phycoerythrin from several species of red algae (Svedberg and Lewis, 1928; Svedberg and Katsurai, 1929; Svedberg and Eriksson, 1932). In a final paper of this series Eriksson-Quensel (1938) found the molecular weight of R-phycoerythrin from *Ceramium rubrum* to be 290,000 between pH 3.0 to 10.0. At other pH values the R-phycoerythrin molecule breaks down into units with a molecular weight of 34,600 or a multiple thereof.

TABLE I

The reduction in optical density (at 540 mμ) of stock pigment solution and crystalline B-phycoerythrin, treated with H_2O_2 for four hours

Per cent H_2O_2	Stock pigment solution $-\Delta E_{540}$	Crystalline B-phycoerythrin $-\Delta E_{540}$
0	0.040	0.000
1	0.874	+0.204
3	1.146	0.000
5	1.060	0.000

In view of the fact that we were dealing with a new type of phycoerythrin it was considered desirable to determine its molecular weight and compare it with that of R-phycoerythrin. The ultracentrifugation experiments were carried out for us through the very kind courtesy of Drs. H. Cook and J. M. Luck of the Department of Chemistry, Stanford University.

B-phycoerythrin that had been recrystallized several times was dissolved at pH 7.0, centrifuged, and dialyzed at 1° C. in 0.05 *M* acetate buffer, pH 5.0. The sedimentation constant ($s_{20} \times 10^{13}$) was determined on the dialyzed preparation in a Spinco ultracentrifuge and found to be 12.0. This value is comparable to that found by Eriksson-Quensel (1938). Consequently it seems very probable that the molecular weights of R- and B-phycoerythrin are the same. The crystalline B-phycoerythrin was judged to be homogeneous from the fact that only one schlieren peak was observed and the absorption boundary of the pigment corresponds almost exactly with it. It may also be noted that Svedberg and Eriksson (1932) determined the molecular weight of what they designated native R-phycoerythrin from *Ceramium* and found it to be similar to the crystalline phycoerythrin from this species.

Electrophoresis

The mobility of crystalline B-phycoerythrin was tested by dissolving it at pH 7.5, centrifuging and then dialyzing at 1° C. in acetate buffer of 0.1 ionic strength, pH 5.0. The movement is toward the anode at the rate of about 2.0×10^{-5} cm.^{2}/sec./volt.

The above value was determined by visually measuring the movement of the ascending and descending boundary in a Tiselius apparatus (no schlieren optics were available). Fairly reproducible results could be obtained, because of the intense color of the pigment.

Crystalline B-phycoerythrin had charge characteristics different from those of the remainder of the pigments in the stock solution and it was possible to determine the pH-mobility characteristics of this pigment. In this case the mobility values were determined from either the ascending or descending boundary, depending upon the pH of the determination. The pH-mobility curve of B-phycoerythrin is presented in Figure 2. No values were determined below pH 5.0 as the pigment tended to precipitate below this pH value. The isoelectric point as extrapolated by this method would be approximately at pH 4.5 which corresponds well with that found for crystallization.

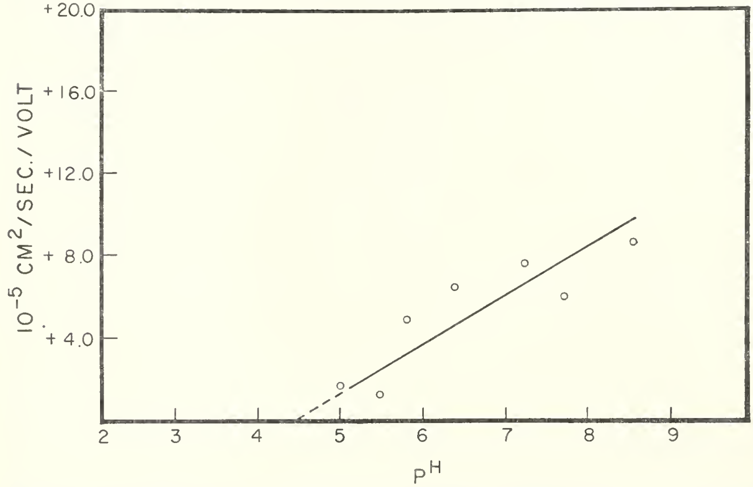

FIGURE 2. pH-mobility curve of crystalline B-phycoerythrin. Mobility in 10^{-5} cm.^{2}/sec./volt. Determinations by visual measurement in Tiselius apparatus.

Other optical properties

The fluorescence spectrum of B-phycoerythrin has been determined by French, Smith, Virgin and Airth (unpublished data); at pH 7.0 the fluorescence maximum is at 578 mμ.

The phycobilin constituents of *P. naiadum* are B-phycoerythrin, phycocyanin and allophycocyanin. At least the two former pigments are photosynthetically active in that they pass absorbed light energy on to chlorophyll. In such studies it is often essential to know the percentage of the total light absorbed by each pigment at various wave-lengths. As purified pigments were available a curve analysis of the absorption of the stock pigment solution was carried out. The percentage of the total light absorbed by each of the phycobilin pigments at their respective absorption maxima is presented in Table II. These values are fairly consistent with those presented by Yocum and Blinks (1954).

TABLE II

Percentage absorption of the total light absorbed by the various phycobilin pigments at different wave-lengths of an extracted pigment solution of Porphyra naiadum

Wave-length $m\mu$	Pigment	Per cent total light absorbed
545	B-phycoerythrin	88
	phycocyanin	9
	allophycocyanin	3
615	B-phycoerythrin	2
	phycocyanin	77
	allophycocyanin	21
655	B-phycoerythrin	0
	phycocyanin	18
	allophycocyanin	82

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SUMMARY

A new phycoerythrin, B-phycoerythrin, isolated from *Porphyra naiadum*, is described. The purification and crystallization, ultracentrifugation, electrophoretic and optical properties of this pigment are discussed. It has a major absorption peak at 545 $m\mu$ with a minor, and transient, one at 565 $m\mu$, which tends to disappear on repeated crystallization. The molecular weight is apparently the same as that of R-phycoerythrin (ca. 290,000). Its iso-electric point is close to pH 4.5, and its mobility (toward the anode) at pH 5.0 is about 2×10^{-5} cm.^{2}/sec./volt.

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HERMAPHRODITISM IN ECHINOIDS[1]

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In his chapter on "Hermaphroditism" Goldschmidt (1923, p. 165) speaks of this as "the most unsatisfactory chapter in the whole sex problem, and up to date our material is insufficient to permit of a correct genetic or physiological understanding." The problem is still further rendered difficult by the fact that it cannot be dealt with experimentally. In the echinoids hermaphroditism, as a rule, is shown by entire gonads of an individual being of one sex or the other, *i.e.,* testes and ovaries in the same animal. More rarely ovarian and testicular tissues develop side by side in the same gonad—an ovotestis. In the five gonads radially symmetrically disposed, all possible combinations have been found, the rarest being the ovotestis.

Such a condition is of sufficiently infrequent occurrence to be noted in the literature in a number of cases in which it has been observed. The paucity of known cases is well expressed by E. B. Harvey (1939, p. 74): "Among the many thousands of *Arbacia punctulata* opened in the course of ten summers at Woods Hole, and many hundreds of *Arbacia pustulosa, Sphaerechinus granularis, Paracentrotus lividus,* and *Parechinus microtuberculatus* opened during several springs at Naples, and many hundreds of *Strongylocentrotus droebachiensis* from Maine, I observed last summer for the first time an hermaphroditic sea urchin, an *Arbacia punctulata* opened on July 4, 1938."

This situation renders an analysis of the phenomenon difficult, and the impossibility of attacking the problem experimentally prevents a precise causative analysis. Nevertheless, an examination of the cases described, the frequency of their incidence geographically and in the classification may give some clue to the phenomenon of what may be called "accidental hermaphroditism." It is hoped that this collection of records may serve to stimulate an interest in the problem and result in further information being published.

At the present time very few statistical records of bisexual echinoids are available. H. B. Moore (1932) reported one hermaphrodite in 3000 *Echinus esculentus* opened during the season 1931–32 at Port Erin. Shapiro (1935) kept an exact account of the *Arbacia punctulata* which he opened during the summer of 1935. He found one hermaphrodite in 2350 animals opened. Albert Tyler (personal communication) at Corona del Mar has kept the most extensive records and reports that 10,000 *Strongylocentrotus purpuratus* opened over a period of several years yielded approximately 20 hermaphrodites, or 1 in 500. This is the highest incidence so far reported. Tyler found that, in addition to normal development as the result of selfing, agglutination of the sperm by autologous sea water was positive. Edward Chambers, working at Berkeley with *S. purpuratus,* in the course of two seasons observed three hermaphrodites.

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At Pacific Grove, although the use of sea urchins has gone on for many years, only one bisexual individual each of *S. purpuratus* and *S. franciscanus* has been detected. In all these cases the selfed eggs gave normal plutei.

In the specimen of *S. purpuratus* opened at Pacific Grove in December, 1950 (A.R.M.), the gonads were swollen with ripe products and easily broken. There were three ovaries, one testis and one ovotestis. None of the eggs could have been fertilized *in corpore*, since no fertilized or segmenting eggs or embryos were seen at the time the animal was opened. However, as soon as the eggs and sperm were free in the sea water, fertilization took place. Development of the selfed eggs was entirely normal, the plutei differing in no way from normals.

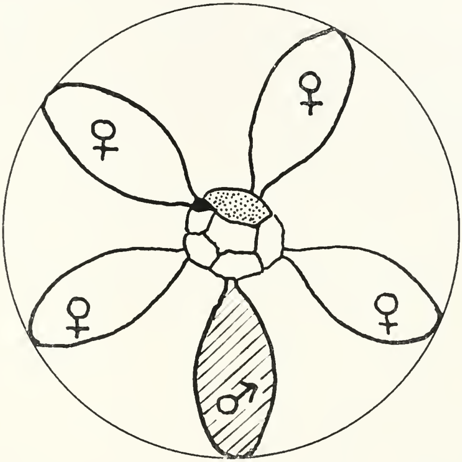


FIGURE 1. Topographical scheme showing details of female and male gonad elements of *S. franciscanus*, viewed from the aboral surface.

In March, 1955, a bisexual individual *S. franciscanus* was found (R. A. B.) at Pacific Grove. This was the first such individual of this species to be discovered. The gonads were separate as to sex, four ovaries, one testis, and no ovotestis. The distribution of the gonads is shown in Figure 1. The selfed eggs produced normal plutei as did the outcrossing of both eggs and sperm. It is to be noted that this is the first case of bisexuality found in this species. The very short breeding season may be a factor.

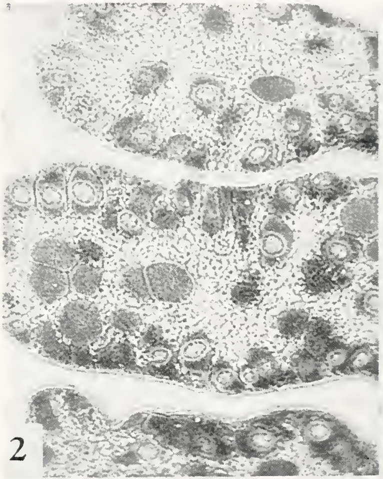
In addition to these examples of sea urchins, several hermaphroditic individuals of *Dendraster* have been found at Pacific Grove. The first was in 1929 (Needham and Moore, 1929), when hundreds of the animals were being used to obtain material for chemical work. In every case the entire gonadal disk was removed and any disk containing white sperm and red eggs would have been detected at once. Since only one was found, it may be assumed that the incidence was of the order of one in 1000. In this one case the eggs and sperm were not fertile *inter se*. The second specimen of *Dendraster* was taken in 1943, and contained both ripe sperm and eggs. The spermaries occupied a little more than half the gonadal disk and yielded abundant sperm. The ovarian half contained a few ripe eggs. Less than half of these were fertilizable with the sperm of the same individual. Such eggs segmented at the normal cleavage rate and gave rise to swimming blastulae in normal time, but the plutei were not vigorous, some larvae remaining blastulae. At 55 hours and 20° C. these selfed larvae were in all stages of juvenility. It is evident that the eggs were defective in their potential, but the sperm was normal since it brought about normal development in normal eggs.

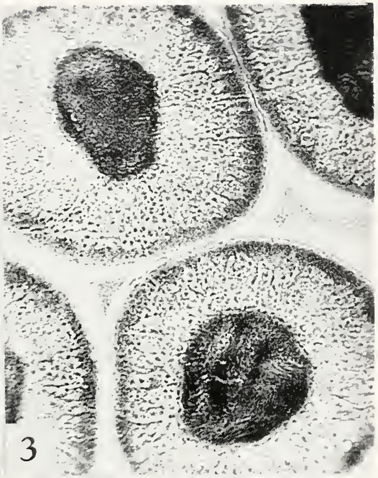
The Ovotestis

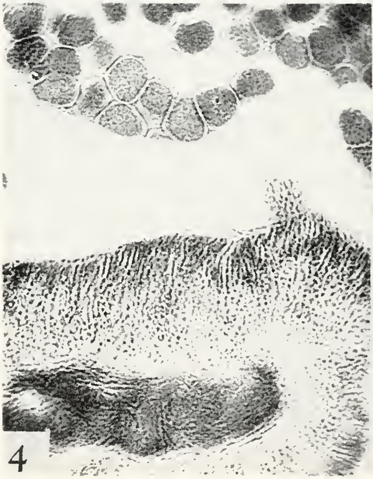
The occurrence of eggs and sperm in the same gonad has been observed in several widely different groups of animals and is a type of accidental hermaphroditism according to Goldschmidt. Ishikawa (1891) described a case in *Gebia major* in which the anterior part of the gonad was testis, the posterior part ovary. The latter was not functional since the eggs were unable to pass out through the vas deferens and consequently atrophied *in situ*. Paul Buchner (1911) described in careful detail the gonads of a bisexual starfish. He observed eggs about the sperm in the testicular vesicles, and ripe sperm infiltrated into the ovary. Harvey (1939) has shown similar conditions in a hermaphrodite *Arbacia*. All of the gonads contained both types of cells, four being mainly female, one predominantly male. Therefore all five gonads were ovotestes. Normal fertilization took place *inter se* and development proceeded to normal plutei. Similar instances have been described by Neefs (1953) and by Reverberi (1947). An account of a hermaphroditic sea urchin *S. pulcherrimus* has been published by Okada and Shimoizumi (1952), in which they give a very complete analysis. One gonad was an ovary, the others were ovotestes. The eggs and sperm did not yield normal larvae when used *inter se*, but gave normal larvae on out-crossing.

As stated above, the hermaphroditic *S. purpuratus* found at Pacific Grove contained one ovotestis. This gonad was preserved and imbedded by Dr. D. P. Abbott at this station. The specimen remained in block until the present season when it was sectioned and mounted by Mr. W. K. Bowen of the Biology Department of Stanford University. The gonad appeared to be divided into an upper and lower half which were, respectively, ovary and testis. Sections were made of parts that were clearly unisexual and of the mixed median zone. Sections of the ovarian half show the ovarian lobes well filled with eggs both ripe and immature (Fig. 2), while sections of the testicular half show normal testicular structure and dense collections of sperm in the vesicles and ducts (Fig. 3). In the median section ovarian and testicular tissue lie side by side, the acini intermingled in the same section (Fig. 4). Ripe ova occur among the sperm (Fig. 5), but no eggs were found fertilized, a fact which presumably was due to the immobility of the sperm. This duplicates the situation in the case described by Harvey.

Two clear exceptions to the general rule that fertilization does not occur *in corpore* before the extrusion of the sex cells from an ovotestis have been noted. H. B. Moore (1935) describes one such case in *Echinocardium cordatum*. In his sketches he figures apparently normal early segmentation stages, morulae and blastulae present in the gonadal ducts of the ovotestis. Reverberi (1947) has described







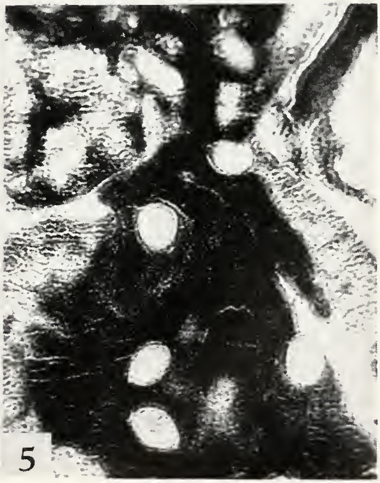


FIGURE 2. Ovarian fraction of ovotestis.

FIGURE 3. Testicular fraction of ovotestis.

FIGURE 4. Boundary zone showing ovarian tissue above, testicular below.

FIGURE 5. From boundary zone showing vesicle with eggs in mass of sperm.

a similar case in *Arbacia pustulosa* at Naples. However, Neefs' (1953) figure of a cell-mass in the lobe of an ovary of a bisexual *Arbacia lixula* is of doubtful significance, for the reason that the structure appears to be a relatively unorganized mass of cells without recognizable embryonic form, and is apparently a solitary instance in the specimen.

Discussion

Various causative factors have been proposed to account for hermaphroditism in echinoids. The oldest is the suggestion of seasonal dimorphism by Giard (1900) who, working at Wimereux, found evidence which he thought sufficient for concluding that *Echinocardium cordatum* is normally a protandrous hermaphrodite, for the reason that in July ova begin to appear in individuals which up to that time he believed had been male. However, Giard's conclusions were later emphatically denied by Caullery (1925) who states that the gonads of this form at Wimereux are entirely quiescent during autumn, the gametes developing during the winter, the phase of maturity beginning in April and ending in August, with a maximum in May. As a result of numerous observations, Caullery says (p. 29): "I have never found a single case of hermaphroditism (in this form) and I cannot explain how Giard could think that at Wimereux, *Echinocardium* shows successive sexuality with protandry, the eggs beginning to appear toward mid-July in the gonads which up to that time were apparently male and full of sperm." Since Caullery, the eminent zoologist and director of the Wimereux Station, has written from the vantage point of 25 years after Giard's paper, it must be considered established that Giard observed a rare case of hermaphroditism in *Echinocardium* (two others have been recorded at Roscoff and one at Port Erin), and that he was clearly in error in postulating seasonal sexual dimorphism for the whole population of this species. Recently Reverberi (1940, 1947) in Italy and Neefs (1937, 1938, 1952, 1953) in France have sought to revive Giard's hypothesis and give it substantial support. Reverberi considers the fact of an ovotestis in itself to be a significant indication of sexual metamorphosis. But to the unbiased worker it is not clear why the fact that normal eggs and sperm occur side by side in an ovotestis, with no evidence whatever of either type of gland degenerating, should indicate a process of sexual metamorphosis. It must be confessed that Reverberi's observations do not give convincing basis for his hypothesis. Neefs has used two lines of argument for her belief in the seasonal change of sex as the basis of bisexuality. One is the presence of degenerate gonads of two colors in an animal. Such a case was described by Gray (1921). We occasionally find them among the *S. purpuratus*. Since these pathological individuals often do not have either eggs or sperm, the more conservative view should be taken, namely, that the appearance is an indication of disease. The other attempt of Neefs to show seasonal sexual dimorphism has been made by means of statistical counts of sex. It must be said that her tables are not convincing, for the reason that at each station a relatively limited number of individuals was examined, and animals of both sexes appeared in each month of the year—a very different picture from that given by Giard. It should also be noted that no one except Giard has reported seasonal incidence of bisexuality.

Recently Egami (1955) has proposed a nutritional basis for bisexuality in fishes. He has shown that periods of starvation, succeeded by food in plentiful

TABLE I

Table of reported cases

Author	Species and cases	Locality
	Normal Eggs and Sperm	
Boolootian (this paper)	*S. franciscanus*	Pacific Grove
Chambers (personal communication)	*S. purpuratus*	Berkeley
Cornman (Harvey, 1956)	*Arbacia punctulata*	Woods Hole
Fisher (Harvey, 1956)	*Arbacia punctulata*	Woods Hole
Fox (Gray, 1921)	*Paracentrotus lividus*	Naples
Giard (1900)	*Echinocardium cordatum*	Wimereux
Harvey (1939)	*Arbacia punctulata* (2)	Woods Hole
Herbst (1925)	*Psammechinus tuberculatus*	Naples
Heilbrunn (1929)	*Arbacia punctulata* (2)	Woods Hole
Herlant (1918)	*Paracentrotus lividus* (12)	Villefranche
Moore, A. R. (this paper)	*S. purpuratus*	Pacific Grove
Moore, H. B. (1932)	*Echinus esculentus*	Port Erin
Moore, H. B. (1935)	*Echinocardium cordatum*	Port Erin
Neefs (1938)	*Paracentrotus lividus* (7)	Roscoff
Neefs (1952)	*Sphaerechinus granularis*	Roscoff
Neefs (1937 and 1953)	*Arbacia lixula* (2)	Banyuls
Reverberi (1940)	*Arbacia pustulosa*	Naples
Tyler (personal communication)	*S. purpuratus* (20)	Corona del Mar
	Defective Eggs—Normal Sperm	
Drzewina and Bohn (1924)	*Paracentrotus lividus*	Roscoff
	Echinocardium cordatum (2)	Roscoff
Moore, A. R. (this paper)	*Dendraster excentricus*	Pacific Grove
	Normal Eggs—Defective Sperm	
Okada and Shimoizumi (1952)	*S. pulcherrimus*	Japan
Viguier (1900)	*Sphaerechinus granularis*	Algiers
Needham and Moore (1929)	*Dendraster excentricus*	Pacific Grove
Shapiro (1935)	*Arbacia punctulata*	Woods Hole
Reverberi (1947)	*Arbacia pustulosa* (5)	Naples
	Psammechinus microtuberculatus	Naples
	No Test	
Chambers (personal communication)	*S. purpuratus* (2)	Berkeley
Gadd (1907)	*S. droebachiensis*	Murmansk
Rulon (personal communication)	*Dendraster excentricus*	Pacific Grove

Genus Incidence—Summary of Cases

Arbacia	15	*Dendraster*	3	*Psammechinus*	2
Paracentrotus	21	*Echinocardium*	4	*Sphaerechinus*	2
Strongylocentrotus	27	*Echinus*	1		

supply, in rare cases result in hermaphroditism. It is possible that other causative factors will have to be taken into account in any final analysis. Two of these are the incidence of hermaphroditism in the classification, and the distribution of the phenomenon geographically. On the first point, it is a striking fact that instances of hermaphroditism so far described are from relatively few genera and species, and these belong almost entirely to the order of the true sea urchins (Table I). Here we find five genera and ten species represented, while the sand dollars and heart urchins furnish examples in one species each. One factor which may in part account for this preponderance of the true sea urchins is the accessibility and extensive use of these animals in scientific work. Most of them are littoral dwellers and are easily obtained, while members of the other two orders as a rule are dwellers of deeper waters and do not yield the quantity of eggs to be found in most of the sea urchins during the breeding season, and hence are little used. In view of the rarity of the phenomenon, the total number of individuals of a species examined is an important factor.

Despite the fact that ten or more species of sea urchins have been extensively used in experimental work, most of the cases of hermaphroditism have come from three genera, namely, *Arbacia, Paracentrotus* and *Strongylocentrotus*. It is notable that *Lytechinus,* which has been extensively used in southern stations, has not yielded a single case. Nor have examples of hermaphroditic sea urchins been reported from northern waters, except for the observation of a specimen of *S. droebachiensis* found fifty years ago at Murmansk by Gadd (1907). No cases have been reported from Scandinavia, none from Maine, none from the Oregon and Washington coasts. The distribution of hermaphroditic echinoids so far reported is in a band between N 35° and 55° in Europe, and between N 32° and 45° in North America.

More information and precise records with publication are needed to give a basis for assessing possible causative factors. It is important that those engaged in work with echinoid material examine animals opened for possible bisexuality, and where this condition is found, a sketch record be made of the positions of ovaries, testes, and ovotestes. Harvey's suggestion that cases of ovotestis may have been passed over as due to contamination when a worker has found occasional fertilized eggs among those freshly shed, is worth bearing in mind. Experiments to determine developmental potentialities both *inter se* and in outcrossing are of great interest and importance.

SUMMARY

Two new cases of hermaphroditism in sea urchins are described. In a search for causative factors of bisexuality, a survey of incidence of the phenomenon in echinoids, both as to genera and geographical distribution, has been made. The suggestion of seasonal dimorphism is rejected.

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THE METABOLISM OF RADIONUCLIDES BY MARINE ORGANISMS. I. THE UPTAKE, ACCUMULATION, AND LOSS OF STRONTIUM ^{89} BY FISHES

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The revolution in biology which occurred about twenty years ago as a result of the utilization of isotopes has led to some problems which could hardly have been foreseen at the time. Biologists can no longer use radioactive material simply as a tool with which to solve specific problems; they are now forced to consider the effects of radioactivity which is introduced beyond their own control into an environment which they are studying. This becomes particularly important to marine and fresh water biologists who are interested in physiological or ecological problems which may be influenced in certain regions by an increase in radioactivity above the background which existed before the first atomic detonation at Alamagordo in 1945.

The weapons testing program of several nations, regardless of the type of blast, has increased the radioactivity of the seas. Underwater detonations, of course, contribute the largest percentage of their radioactivity directly to the water, but most of the fall-out from aerial bursts can be expected to appear ultimately in the ocean, since the land area of the earth is only about 30 per cent of the total. Run-off from the land will also increase the radioactivity of the seas. Far more important than the radioactivity which appears as a result of weapons testing, however, is the radioactivity which inevitably will be introduced into the oceans from nuclear power plant wastes and atomic-powered ships.

There are several important reasons for studying the metabolism of fission products and other radionuclides in marine organisms. First, several fission products are known to be potential hazards from a public health standpoint (N.B.S. Handbook 52). Second, almost nothing is known about the metabolism of these radioelements by marine species. Third, we cannot tell at present what potential ecological effects may be brought about through the deleterious action of radiation on the marine biota, but the possibility exists that some adverse changes, such as those which apparently occurred in White Oak Lake (Krumholz, 1956), might occur in estuaries and other inshore regions. It is therefore important to study these problems now, before the oceans become polluted with radioactivity, because the changes which may occur will be irreversible at least for several centuries.

The problems raised by the above considerations can best be solved by studying the metabolism of these radionuclides not only in individual organisms, but also in relation to the various trophic levels by way of the food chains. As desirable as such studies may be, it is impossible to undertake investigations of this magnitude

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for the entire marine biota. Thus, because of our special facilities at the Hawaii Marine Laboratory, we have confined our research to fishes which are representative of three distinct marine habitats, and include herbivores belonging to the second trophic level, and carnivores from the third and fourth trophic levels.

Strontium was selected for our initial studies for two reasons. First, it is chemically similar to calcium, and is therefore a "bone seeker." As such, if radioactive, it may interfere with the blood cell formation of many animals. Second, Sr^{90} has a half-life of about 28 years, so that the deposition of an atom of Sr^{90} into a tissue which has a slow rate of turnover may result in radiation exposure for the entire life of the animal. These characteristics make radiostrontium a particularly hazardous fission product. In these experiments we have used Sr^{89} because of its much shorter half-life ($\sim$ 53 days) which decreases the danger of contamination in the laboratory.

The particular objective of the present study was to measure the uptake, accumulation, and loss of radiostrontium by the various tissues and organs of selected species of fish when the isotope was given orally, by intramuscular injection, and by the immersion of the fish in sea water enriched with Sr^{89}.

Materials and Methods

The large pelagic fishes used in these experiments consisted of the black skipjack (*Euthynnus yaito*), the yellowfin tuna (*Neothunnus macropterus*), and the so-called dolphin (*Coryphaena hippurus*). These species are fast-swimming, wide-ranging carnivores which occupy the fourth trophic level. Among the small fish used, the papio (*Carangoides ajax*), and the aholehole (*Kuhlia sandvicensis*), are small carnivores common along the reefs and shores in the Hawaiian Islands and occupy the third trophic level primarily. The aholehole is also able to adapt itself to brackish water environments, and has even been found well into fresh water streams (Tester and Takata, 1953; Tester and Trefz, 1954). The third small species used, *Tilapia mossambica,* is a sluggish fish, predominantly herbivorous, but facultatively omnivorous, and may be placed in the second trophic level. It prefers brackish water, but is well adapted to either fresh water or sea water.

Carrier-free $strontium^{89}$ was obtained from Oak Ridge and fed to the large fishes by filling gelatine capsules with cracker crumbs and a measured quantity of the isotope solution. The capsule was sewn into a small piece of fish muscle which was held just under the surface of the water by a weak thread. As the fish swallowed the bait, the thread was broken off. In this way the capsule could be given to a particular fish. In some instances small fishes were force-fed a gelatine capsule prepared in the same way. Others were fed by incorporating a measured amount of Sr^{89} into a gelatine solution which was allowed to solidify in a small plastic tube. The tube was put into the fish's stomach and the gelatine was extruded with the aid of a syringe. A dose solution was prepared by extruding the same quantity of radioactive gelatine into a volumetric flask.

The large fish were perfused with a mixture of two parts sea water and three parts distilled water. The brain, eyes, spinal cord and integument were removed, and the excised internal organs were further soaked in distilled water until no blood was apparent in the water. All the rinsings were added to the blood. The gut was opened, and any material remaining in it was flushed out. Only the eyes and the visceral organs were removed from the small fish which were not perfused.

The remainder of the fish, consisting of muscle and skeleton for the large fish, or muscle, skeleton and integument for the small fish, was then put in a pressure cooker, brought to 20 pounds pressure and allowed to cool. After this treatment, the muscle was easily removed from the bones, and any flesh remaining on the gill arches was removed with warm formamide. Control experiments indicated that no leaching or loss of strontium occurred as a result of the pressure cooker treatment.

Wet weight of organs was obtained without blotting, and the tissues were dried at about 110° C. for 48 hours. The dried tissues were put in a muffle furnace which was brought to about 550° C. The furnace was then shut off and the samples left overnight. A slow stream of air was introduced in the oven to aid combustion.

The ash was ground and spread evenly on aluminum planchettes with the aid of water and a detergent, and dried under infra-red lamps. The samples were counted in triplicate when possible, using commercial counters and scalers. A minimum of 2560 counts was taken on each sample, including background. No corrections were made for back-scatter, self-scatter, or self-absorption. The latter is very small at the ash densities which were used (< 5 mg./cm.^{2}). The scalers were calibrated daily with Bureau of Standards nuclides, and the only correction applied was for radioactive decay. The counts/minute of the samples were compared with aliquots of the actual dose given in each instance. Specific details for each experiment will be described at the appropriate place.

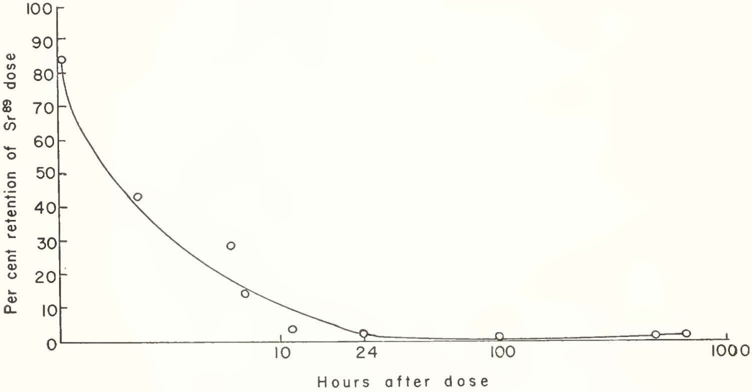

FIGURE 1. The decrease of ingested Sr^{89} in pelagic fishes as a function of time.

RESULTS AND DISCUSSION

A. *Ingestion of Sr^{89} by large pelagic fishes*

Figure 1 shows that the excretion of a single dose is very rapid: about 50 per cent disappeared within a few hours, and only 1–2 per cent was left after 24 hours. This latter value persisted for the remainder of the experiment which lasted 27 days.

Table I shows the distribution of the Sr^{89} in the various organs and tissues of these fishes, and Figure 2 is a graph showing some of these data. This graph is presented as the percentage of radioactivity of the different organs and tissues in terms of the total radioactivity found in the entire fish when it was killed. It is apparent that the tissues are segregated into two groups with regard to strontium retention: the visceral, and the structural. The visceral organs and tissues, including the blood, kidney, foregut, midgut, hindgut, spleen, liver, caecum and heart, show a continuing decrease in radioactivity beginning one hour after the administration of the dose. The structural tissues, including the skeleton, head and

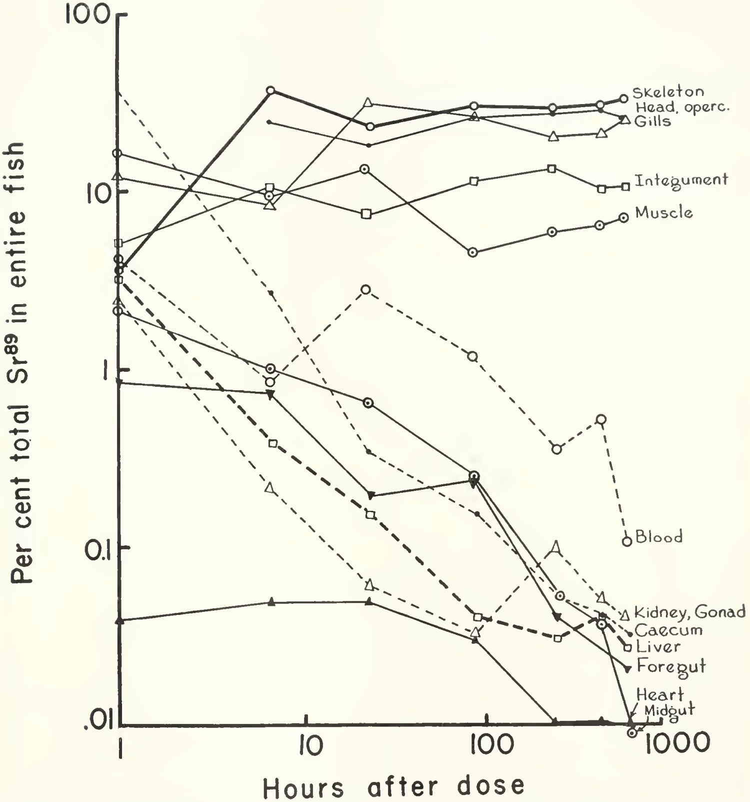

FIGURE 2. The distribution of a single ingestion of Sr^{89} in organs and tissues of pelagic fishes as a function of time.

opercular bones, gill arches, integument and muscle appear to concentrate strontium rapidly to a level which is maintained more or less constant for a relatively long period of time. The turnover or excretion of strontium in these structures is therefore slow.

TABLE I

Accumulation of ingested Sr⁸⁹ in the various organs and tissues of pelagic carnivorous fishes expressed as percentage of total activity

Tissue	Species	EY[1]	EY	EY	CH[2]	EY	EY	EY	NM[3]	EY	EY
	Dose in μc	5.55	480	240	51.0	240	464	464	464	371	371
	Duration in hours	1	$2\frac{1}{4}$	6	7	$11\frac{1}{2}$	24	96	264	480	648
Heart		0.04	0.01	0.03	0.049	0.11	0.05	0.028	0.01	0.014	0.007
Gall bladder		0.05	0.04	0.07	0.10	0.08	0.03	0.0001	0.01	0.004	0.002
Blood		4.21	6.68	15.00	0.85	8.07	2.73	1.14	0.35	0.51	0.12
Gill flesh		12.44	0.18	0.91	8.56	5.06	30.61	25.72	2.42	1.42	2.21
Gill bone			1.34	6.47		26.39			16.80	19.48	22.76
Caecum		37.01	7.67	7.84	2.70	2.64	0.34	0.15	0.05	0.04	0.029
Foregut		0.89	9.32	1.12	0.74	1.03	0.20	0.24	0.04	0.04	0.018
Midgut		2.28	14.16	16.50	1.08	1.48	0.65	0.25	0.05	0.036	0.003
Hindgut		11.78	3.98	21.26	2.26	0.11	0.15	0.024	0.03	0.015	0.016
Gut contents		—	48.32	12.73	0.056	19.65	—	0.10	0.013	—	0.0008
Head, operculum		—	0.41	1.09	24.99	6.28	18.33	24.58	28.18	29.91	24.58
Appendicular skeleton		3.60	0.40	1.19	36.21	8.45	23.69	30.32	29.15	30.47	31.43
Liver		3.34	1.48	3.04	0.39	2.46	0.15	0.04	0.03	0.04	0.027
Spleen		0.20	0.32	1.39	0.08	0.60	0.03	0.008	0.03	0.010	0.003
Tail		—	0.42	0.15	—	0.00	—	—	—	—	—
Brain, spinal cord		0.23	0.00	0.01	1.24	0.05	1.66	1.70	1.33	0.030	0.004
Eyes			0.04	0.06		0.60			2.02	1.34	1.34
Integument		5.28	1.69	0.86	10.20	5.89	7.69	11.37	13.73	10.25	10.51
Integument flesh (aliquot)		—	0.01	0.01	—	0.05	—	—	—	—	0.065
Integument scales (aliquot)		—	0.02	0.02	—	0.11	—	—	—	—	0.091
Gonad		2.40	0.09	0.47	0.22	0.08	—	0.004	0.03	0.023	0.020
Kidney			0.08	0.16		0.09	0.06	0.027	0.07	0.035	0.022
Light muscle		16.23	3.23	8.74	4.19	10.01	12.84	3.94	5.26	5.69	5.79
Dark muscle			0.10	0.86	5.25	0.70	0.72	0.48	0.47	0.63	0.95

[1] EY = *Euthynnus yaito.*
[2] CH = *Coryphaena hippurus.*
[3] NM = *Neothunnus macropterus.*

We do not interpret the departures from a smooth curve for any one organ to indicate a sequential pattern. In other words, a rise in the radioactivity of one organ and the fall of radioactivity in another are not necessarily linked by way of precursor relations. Each point on the graph represents a single fish, and individual differences can most likely account for the small deviations of the curves.

In order to study the sequential pattern of strontium metabolism, a much larger group of fish would have to be used, particularly since it is known that there is a very large difference in the time a food bolus remains in a fish as compared with another fish of the same species living in the same tank.

The rank order of radioactivity in the organ systems of these fishes is: skeleton, gills, integument, muscle and viscera. It is interesting to note that the dark muscle, which has a better supply of blood, has less radioactivity/gram ash than has the light muscle. Similarly, the "specific activity" of the gills, that is, the counts/minute/mg. ash, was considerably higher than that of the axial skeleton. Goldberg (personal communication) has analyzed yellowfin tuna for various metals, and found that the gill arches and filaments had considerably more strontium in them than had the bones. His values for strontium in the gills would therefore be a minimum value, since any flesh adhering to the sample would tend to dilute the strontium concentration. The main chemical difference between the gill arches and the remaining skeletal tissue is the presence of cartilage in the gill rakers. In our own work, and in the work of others (Jones and Copp, 1951), there is a suggestion that cartilage may have a greater capacity to exchange ionic calcium for strontium than has bone. For example, we have found a higher "specific activity" in the eye, which has cartilaginous ossicles, than we have found in skeletal bone. Moreover, Jones and Copp found that the uptake of strontium by the skeleton is more rapid in young rats than it is in adults. It is possible that the explanation of these differences might lie with an increased amount of Sr^{++} binding by the protein of the cartilage as compared to that bound by the protein of calcified bones. Perhaps differences in the amount of blood supplied to ossified and cartilaginous tissue, or some other properties of cartilage may also be involved.

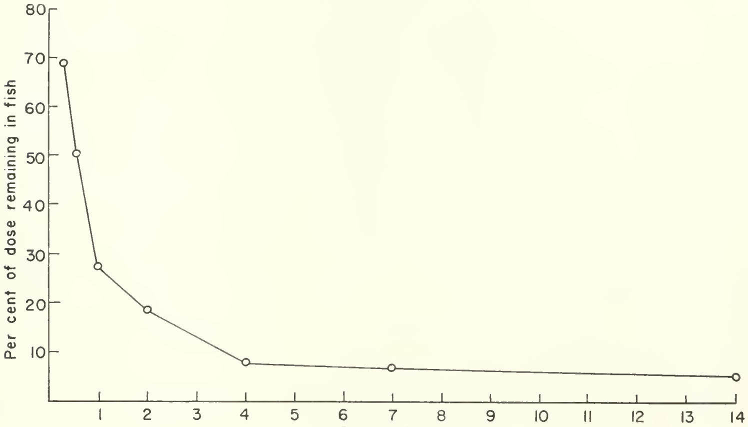

FIGURE 3. The decrease of ingested Sr^{89} in *Tilapia mossambica* as a function of time.

TABLE II

Percentage of total radioactivity recovered in various organs of Tilapia mossambica. (Each horizontal column represents an individual fish given a dose of 20 μc)

Duration	Tissue					
	Integument	Eyes	Visceral organs	Gills	Muscle	Skeleton
2 hr.	10.58	0.26	31.88	28.02	9.44	19.81
	1.73	0.08	88.01	1.43	0.83	7.92
4 hr.	12.54	0.36	28.64	23.50	6.45	28.52
	0.32	0.07	65.81	11.34	10.88	11.58
	1.70	0.29	24.41	36.18	6.09	31.34
8 hr.	10.64	1.38	44.50	6.90	3.72	32.85
	8.01	0.24	44.40	15.83	5.46	26.06
	19.66	1.62	11.99	17.10	1.74	47.88
12 hr.	23.07	0.24	42.34	3.65	7.61	23.08
	4.63	1.26	78.36	1.95	1.61	12.19
	7.86	0.67	40.92	8.94	4.26	37.36
24 hr.	25.67	0.32	1.45	9.43	8.96	54.18
	24.93	0.36	1.60	8.60	3.52	60.99
	28.31	0.53	3.57	8.74	4.82	54.03
48 hr.	22.67	0.31	0.62	4.81	1.62	69.97
	5.79	0.83	3.32	14.35	7.39	68.32
	26.48	0.29	1.76	8.40	2.30	60.77
4 days	23.10	0.16	0.40	5.99	2.17	68.17
	26.71	0.27	0.64	8.08	2.38	61.92
	23.63	0.17	0.93	8.20	3.35	63.72
7 days	21.88	0.22	0.97	8.02	2.26	66.65
	20.84	0.21	0.57	8.28	1.92	68.18
14 days	31.42	0.24	0.17	8.92	1.97	56.73
	29.63	0.41	1.10	10.06	2.10	56.69
	20.39	0.22	0.51	8.07	1.24	69.57

B. *Ingestion of Sr^{89} by Tilapia*

Figure 3 shows that the rate of excretion of Sr^{89} by *Tilapia* is much slower than that by the pelagic fishes. About 50 per cent is still present after one day, and the time required to reach a more or less constant level is about four days. The amount which persists is also larger than that observed with the pelagic fishes, although the variability among the *Tilapia* was fairly large. The *Tilapia* used in these experiments were fed the Sr^{89} in gelatine capsules containing cracker crumbs. Occasionally crumbs were observed in the carboys used to hold three of the experimental fish, and therefore the true dose could not be ascertained. The incorporation of the isotope in gelatine for the later experiments has apparently obviated this difficulty.

Table II presents the data concerning the percentage of the total radioactivity recovered in the various organs and tissues. Figure 4 is a graph of this information except that the ordinate is given in microcuries/gram fresh weight of fish.

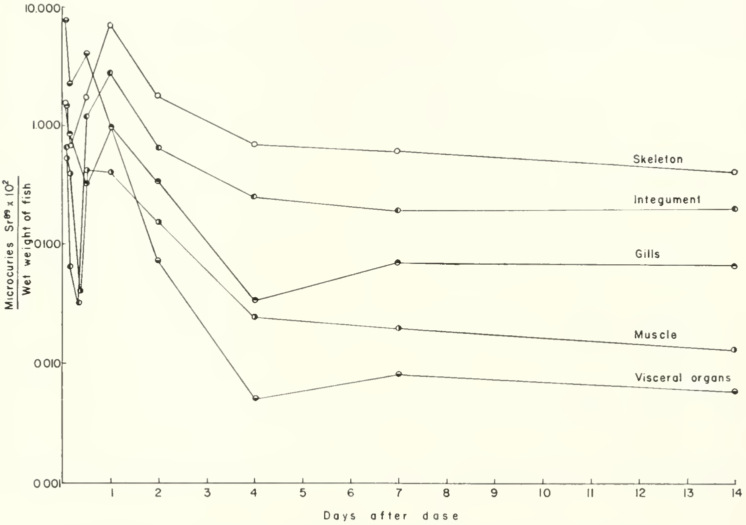

FIGURE 4. The distribution of a single ingestion of Sr^{89} in organs and tissues of *Tilapia mossambica* as a function of time.

The structural tissues account for the bulk of the radioactivity where approximately 90–95 per cent of the activity is present in both *Tilapia* and the pelagic fishes. Roughly, about 60 per cent is accounted for by the skeleton, 30 per cent by the integument, 10 per cent by the gills, 2 per cent by the muscle, and 1 per cent by the viscera. A larger percentage of radioactivity is found in the integument of *Tilapia* than in the integument of the other fishes, probably because a larger percentage of the body weight of this species is due to the large scales. The percentage of the total radioactivity in *Tilapia* was found to decrease in the following order: skeleton, integument, gills, muscle and viscera. The order in the pelagic fishes studied was skeleton, gills, integument, muscle and viscera.

C. *The ingestion of Sr^{89} by aholehole*

Five aholehole were each fed 48 μc of Sr^{89} in gelatine capsules and kept in running sea water 24 hours before killing. The entire fish was then dried and ashed, and Table III shows that the results are much more reproducible than they were using *Tilapia.* After 24 hours the latter fish retained approximately the same percentage of the dose as did the aholehole, but the range was between 2 and 20. No further experiments were conducted with aholehole at this time, but because of their apparent superiority as a laboratory animal we plan to use them in experiments which will be reported at a later date.

Although we have completed some experiments on the repetitive feeding of Sr^{89}, the uncertainty of the exact dose in some instances, and the excessive range of retention by *Tilapia* during short periods have caused us to omit these data here. The results of such experiments on other fish will be reported at a later date.

TABLE III

Percentage of Sr^{89} retained by aholehole 24 hours after ingestion

Fish	Per cent of dose
1	10.43
2	7.42
3	9.16
4	5.70
5	7.97
Aver.	8.13

D. *The uptake and accumulation of Sr^{89} injected intramuscularly into tuna and Tilapia*

Because our results showed that the biological half-life [3] of strontium in fish muscle fell in the same range as that found for the bones and integument, rather than with the soft tissues as was expected, additional experiments were devised to study the retention of Sr by muscle. These experiments also enabled us to study the metabolism of radiostrontium which was introduced by a method other than feeding.

A yellowfin tuna was injected with 288 μc of Sr^{89} at the base of the pectoral fin. The fish was killed and analyzed after it had been in running sea water for 19 hours. A comparison of the percentage of Sr^{89} recovered in the organs and tissues of a tuna which received the isotope by injection with that of a tuna receiving an oral dose (Table IV) shows that the digestive organs of the latter were relatively more radioactive than were the corresponding organs of the injected fish. This result is perhaps to be expected, but there are several other outstanding differences in the distribution of the isotope within this time period. The muscle tissue of the fish receiving the injection had almost three times the percentage of Sr^{89} as had the muscle of the fish receiving the radioisotope orally. The gills of the former, on the other hand, had only about half the percentage of Sr^{89} as had the orally dosed fish. The percentage of radioactivity in the integument and the skeleton of both fish was about the same, and it is not possible to state whether or not the differences found in the remaining organs are significant.

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[3] We define the term "biological half-life" in this paper to mean the time required for half the labelled strontium to be removed from the tissue or animal in question, exclusive of radioactive decay.

The fact that such a large percentage of the Sr^{89} was retained by the muscle suggested that it might be informative to study this process over a longer period of time. Thus, *Tilapia* were injected intramuscularly between the caudal and anal fin with Sr^{89} Cl$_2$ neutralized to about pH 6. The fish were kept in aerated, but not circulating, sea water for the first 24 hours, during which time samples of the water in the aquaria were removed. After 24 hours the fish were put in running sea water and removed at intervals. Figure 5 shows that the radiostrontium was rapidly excreted for about the first twelve hours, and that the rate decreased thereafter.

Table IV

A comparison of the percentage of Sr^{89} recovered in the organs and tissues of tuna related to the route of administration

Tissue	Oral[1]	Injected[2]
Integument	7.69	7.15
Gill bones	30.61	15.25
Gill flesh		1.81
Head, operculum	18.33	14.41
Appendicular skeleton	23.69	23.25
Light muscle	12.84	33.24
Dark muscle	0.72	1.51
Foregut	0.20	0.11
Midgut	0.65	0.36
Hindgut	0.15	0.07
Kidney/gonad	0.06	0.20
Heart	0.05	0.04
Caecum	0.34	0.16
Liver	0.15	0.13
Spleen	0.03	0.02
Gall bladder	0.03	0.37
Blood	2.73	1.79

[1] Skipjack, duration 24 hours.

[2] Yellowfin, duration 19 hours.

An analysis of the percentage of the dose retained by the fish confirmed the fact that after 24 hours comparatively little strontium was excreted, and that most of the strontium remaining was held by the fish for the duration of the experiment. These results are shown graphically in Figure 6. The points on the graph represent the average of three fish. The greater retention of the five-day fish as compared to the one-day fish can be ascribed to individual variation. The range of retention between the one- and the 21-day fish is of the same order of magnitude as the range of retention at any single time interval. In other words, the curve, neglecting individual differences, is very likely parallel to the abscissa.

The internal distribution of the injected Sr^{89} requires longer to reach a "levelling-off" than does Sr^{89} given orally (Fig. 7). In the former instance, the time required is about one week, whereas in the latter instance the "levelling" occurs within two days. In both situations, however, the percentage of the total radioactivity retained by each of the organ systems is the same.

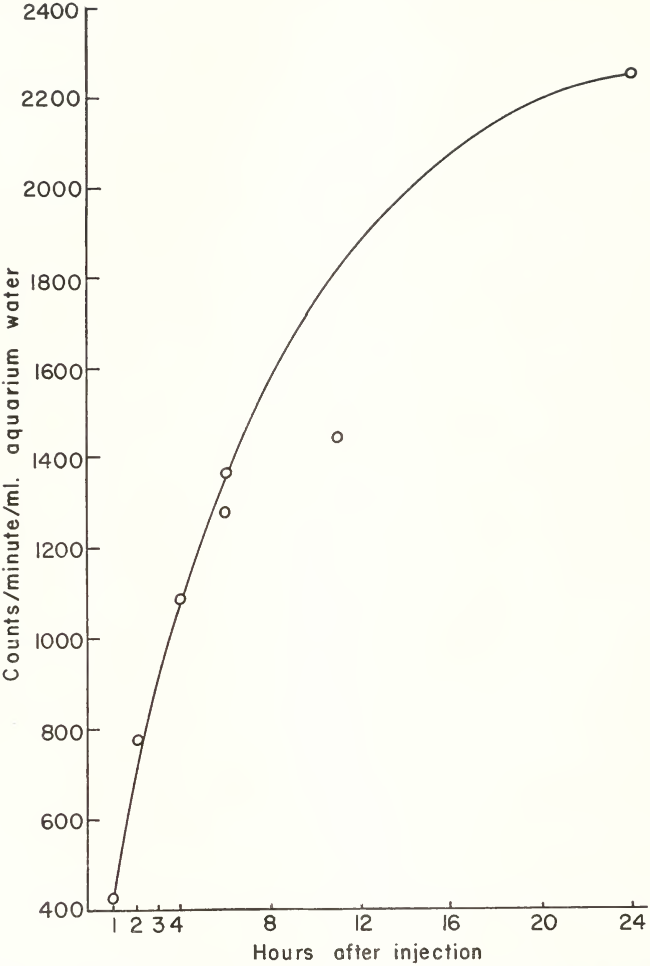

FIGURE 5. The rate of excretion of Sr^{89} injected intramuscularly into *Tilapia mossambica*.

The aquaria used in these experiments were inverted five-gallon carboys with the bottoms removed. Feces and other solid material thus settled to the neck of the carboy and could be removed through glass tubing which just penetrated the rubber stopper. In this way the feces were removed from the tanks six times during the first 24 hours at each sampling of the tank water. The average total

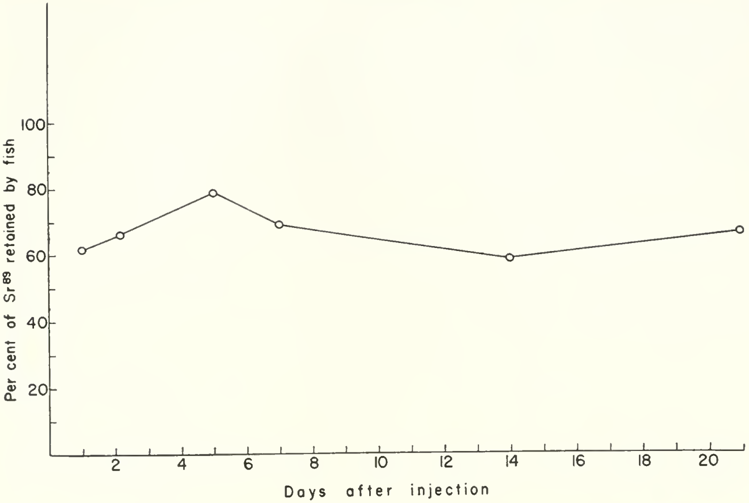

FIGURE 6. The continuing retention of Sr^{89} injected intramuscularly into *Tilapia mossambica.*

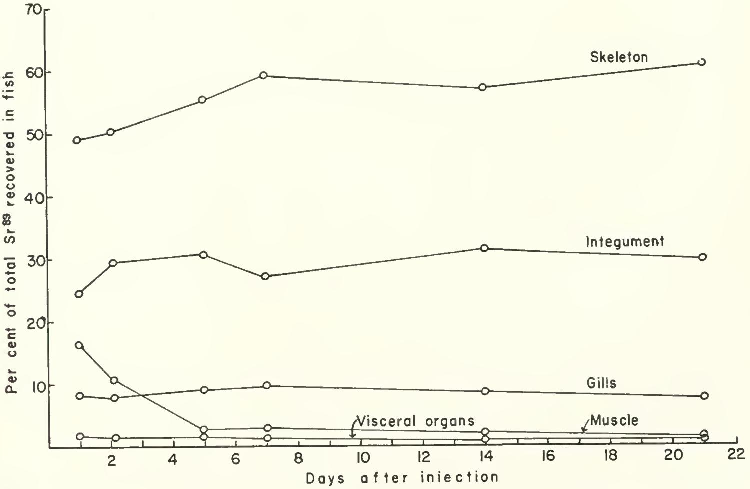

FIGURE 7. The internal distribution of Sr^{89} injected intramuscularly into *Tilapia mossambica.*

Sr^{89} recovered in the feces was 0.35 per cent of the dose at 24 hours, but the amount of leaching is unfortunately unknown.

The injected fish retained a much greater percentage of the dose than did the fish which received the strontium orally. These results suggest that the injected Sr^{89} was actually absorbed to a greater extent, and that the absorption of strontium through the gut is not efficient. Slow, continuous diffusion from the site of injection may also allow a larger amount to become incorporated into the various tissues. If the rate of blood supply to the muscles is less than that to the visceral organs, one might expect the muscle to retain the strontium for a comparatively longer period. The fact that the dark muscle of tuna retained much less strontium than did the light muscle (Table I), and the fact that the dark muscle is better supplied with blood than is the light muscle, suggest that the degree of vascularization is of some importance. One might therefore reasonably expect the cartilaginous tissues, such as the gill rakers and gill rays, to retain the strontium for longer periods than does the bone which is better supplied with capillaries.

Although the amount of strontium in tuna muscles and visceral organs is of the same order of magnitude (Goldberg, personal communication), some specific binding of strontium may occur with muscle protein which does not occur with the proteins of the visceral organs. Further, the very long biological half-life of strontium in the muscle suggests that fish muscle may not be in such a "dynamic state" as one ordinarily assumes according to the researches of Schoenheimer and his associates (Schoenheimer, 1942). Moreover, there is evidence that mammalian muscle protein has a very much slower turnover rate than has the proteins of the visceral organs (Tarver and Schmidt, 1942). Therefore, the slow turnover of strontium in the fish muscle may be only a reflection of the slow turnover of muscle in general.

E. *The uptake and accumulation of Sr^{89} in solution by Tilapia*

Because the pattern of distribution of Sr^{89} in the tissues and organs of several species of fish appears to be similar, both when the isotope was given orally and by injection, one might extrapolate and conclude that regardless of the mode of entry, the internal distribution of Sr^{89} ultimately would be the same. However, to secure more information on this point, and to ascertain whether or not fish could take up strontium directly from the sea water, a situation which is possible in nature, the experiments described below were carried out.

Six *Tilapia* were put into each of four tanks containing 20 liters of filtered sea water and 1744 μc of Sr^{89}. The water was aerated during the experiment, but the fish were not fed. The total amount of Sr^{89} available during the experiment can be considered constant, since even at 21 days, the total Sr^{89} removed by six fish in a tank was less than one per cent of the available dose.

Figure 8 shows the rate of uptake from solution in terms of microcuries of Sr^{89}/gram fresh weight of fish. The rate slows down considerably after about a week, but uptake is apparently still continuing. The ordinate on the right indicates that within 21 days, the ratio of internal Sr^{89} to external Sr^{89} is still less than one.

The internal distribution of the Sr^{89} taken up from solution is shown in Figure 9. The Sr^{89} found in the skeleton is about 40 per cent as compared with a value close to 60 per cent when the strontium is fed or injected. The amount found in the integument in all cases is about the same, and the gills and muscles show little variation. The amount found in the visceral organs, however, is markedly different. The individual organs were not ashed and counted separately because of their very small size, so it is not possible to state in what organ or organs the very large percentage of Sr^{89} was located.

Since marine fish in general swallow more water than do fresh water fish to maintain proper osmotic balance, it is possible that the principal route of entry of the isotope in solution is by way of the gut. However, our direct feeding experiments with a variety of fishes indicate that strontium is rapidly eliminated from all the visceral organs. How is it, then, that so much strontium remains in the visceral organs when the fish is immersed in the isotope? A probable explanation is that the fish is being fed the isotope continually, in effect, every time it swallows. Since the concentration of Sr^{89} was found to be higher in the sea water than in the fish, only a small amount of sea water present in the gut would account for the large percentage of total Sr^{89} which was found in the visceral organs. The concentration of Sr^{89} in the sea water was 8.7×10^{-2} μc/ml. Assuming arbitrarily that 50 per cent of the total Sr^{89} of the fish was in the visceral organs (Fig. 9), this amount equals about 12×10^{-3} μc/gram fresh weight of the organs. If only from 0.1–0.2 ml. of sea water was present in the gut, this would account for the radioactivity

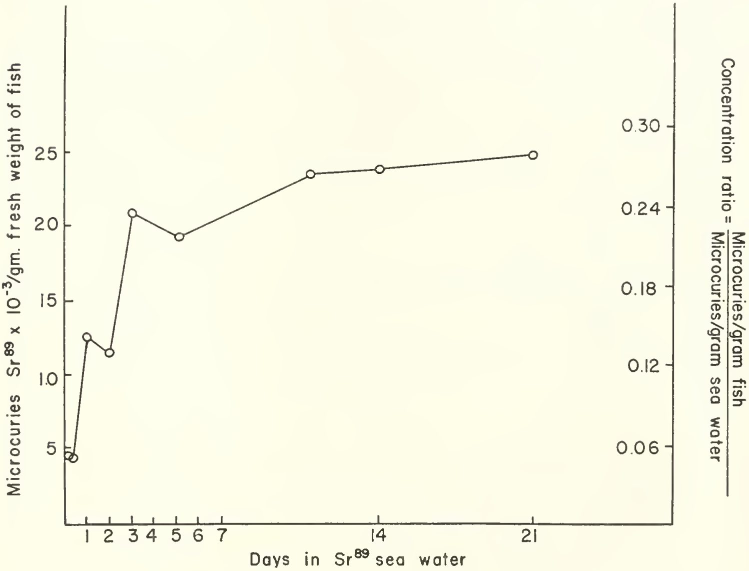

FIGURE 8. The uptake of Sr^{89} in solution by *Tilapia mossambica*, expressed as the concentration ratio.

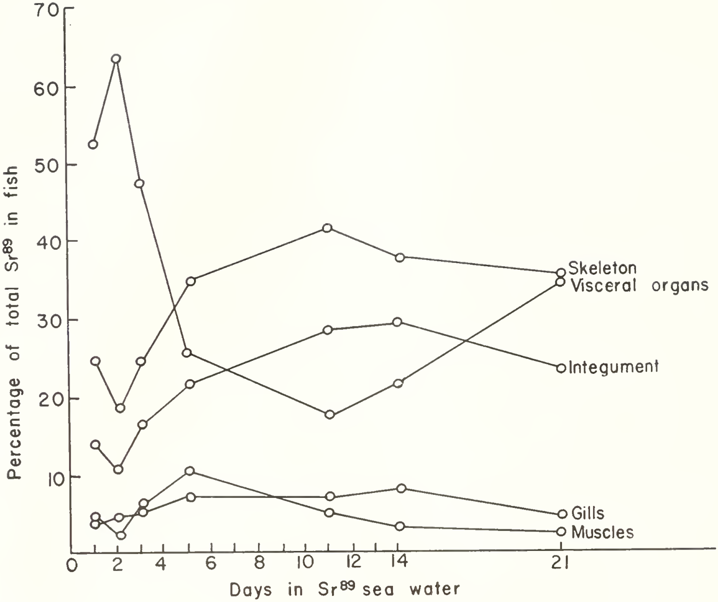

FIGURE 9. The internal distribution of Sr^{89} taken up by *Tilapia mossambica* from sea water.

found. What is important, then, is the rank order of radioactivity in the various organs and systems. Excluding the visceral organs for the reasons given above, it is seen that the other organ systems studied fall in the same rank order regardless of the mode of entry of the radiostrontium.

SUMMARY

1. The ingestion of Sr^{89} by large pelagic fishes results in the excretion of most of the isotope in a few hours. The small percentage remaining after one day persisted for the 27 days of the experiment. The strontium is rapidly eliminated from the visceral organs and tissues, but the structural tissues, including the bones, gills, integument and muscle, maintain their strontium level more or less constant. The turnover of strontium in these latter tissues is therefore slow.

2. Dark muscle, which has a better blood supply than light muscle, retains less Sr^{89}. Similarly, bone, which is better supplied with blood than is cartilage, retains less Sr^{89} than the gill arches or the cartilaginous eye ossicles.

3. The excretion of Sr^{89} by *Tilapia mossambica* is much slower than it is by the pelagic fishes. The percentage of the dose retained is somewhat larger, and most of the radioactivity is found in the structural tissues.

4. About three times as much Sr^{89} was found in the muscle of an injected tuna as compared with another fish receiving the isotope orally. The gills of the former fish had only about half the activity found in the latter. From 60–70 per cent of the dose injected into *Tilapia* muscle was retained by these fish for 14 days. The long biological half-life of Sr^{89} in fish muscle is contributing evidence for the slow turnover of muscle tissue in comparison with such tissues as liver or kidney.

5. *Tilapia* were able to concentrate Sr^{89} directly from the sea water, although the ratio of Sr^{89} in the fish to the Sr^{89} in an equal weight of sea water was only about 0.3 after three weeks. Except for the visceral organs, the rank order of the retention of radioactivity in the various tissues is skeleton, integument, gills and muscle. This is the same distribution as was observed after oral administration of Sr^{89}. Because marine fish swallow water continually, a small amount of water in the gut might account for the relatively large percentage of radioactivity found in the viscera.

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THE METABOLISM OF RADIONUCLIDES BY MARINE ORGANISMS. II. THE UPTAKE, ACCUMULATION, AND LOSS OF YTTRIUM^{91} BY MARINE FISH, AND THE IMPORTANCE OF SHORT-LIVED RADIONUCLIDES IN THE SEA[1, 2]

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A study of the metabolism of radioyttrium is important for two reasons: first, yttrium^{90} occurs as the daughter of long-lived strontium^{90}, an element which has considerable interest from the standpoint of public health, and second, yttrium^{91} occurs as a direct fission product to the extent of about four per cent of the total radioactivity present in a fission product mixture one year old. Fission products are being introduced into the seas to a small extent as a result of fall-out, and also from nuclear reactor plants located near the seaboard. The latter situation now occurs in the Irish Sea near Harwell, and more activity of this type may occur as reactor plants increase in number. Spooner (1949), in a noteworthy study on the metabolism of radioyttrium by marine algae, showed that this element was capable of being concentrated by certain algae, and thus stimulated the interest of marine biologists to learn more about the metabolism of the nuclide of mass^{90}.

Because we had begun experiments on the metabolism of strontium^{89} and strontium^{90}, both of which contain a certain amount of yttrium^{90}, we were interested in comparing the metabolism of these two elements in marine fish. Although the ratio of Sr^{90}: Y^{90} in an equilibrium mixture is about 3000:1, it was conceivable that some of the results we obtained might have come from the small amount of yttrium present.

In a number of instances the concentration of certain elements is sometimes greater within a marine organism than it is in the surrounding sea water (Noddack and Noddack, 1939; Vinogradov, 1953). For example, one cannot predict, let alone explain, why one species of green algae will accumulate yttrium almost exclusively from a mixture of strontium and yttrium, while another accumulates strontium exclusively. Thus, Rice (1956) reports that *Carteria* sp. takes up 100 per cent of its radioactivity from the strontium in a Sr^{90}–Y^{90} mixture, but *Chlorella* sp., which is also a member of the Chlorophyceae, has 95 per cent of its radioactivity in the form of yttrium. Such tremendous differences in accumulation cannot be explained on the basis of size, that is, surface per gram protoplasm. Rather, the explanation is more likely to be one involving the chemical nature of the surfaces among the different algae. Because closely related algae are able to concentrate one element more than another, it is not unreasonable to suspect that one organ of a fish might have a greater avidity for yttrium than it has for strontium. Unfor-

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[2] Contribution No. 83, Hawaii Marine Laboratory, University of Hawaii.

tunately, the small size of the fish used in these experiments did not permit us to separate the visceral organs in detail, so that we can only report the amount of radioactivity found in the integument, skeleton, gills, muscle, and in the combined visceral organs.

Materials and Methods

Yttrium^{91}, obtained from Oak Ridge, was incorporated into a two per cent gelatine solution, and 0.5 ml. was drawn up into a piece of Tygon tubing of small diameter. When the solution solidified, it was extruded directly into the fish's stomach with the aid of a syringe. The dose was 5.5 microcuries. The fish used were *Tilapia mossambica,* each of which weighed about 100 grams. After the radioyttrium was administered, three fish were put into a single carboy with 20 liters of sea water which had been filtered through a No. 4 Mandler filter. Four carboys were used for the four time intervals of 1, 2, 4 and 14 days. The water was aerated, but the fish were not fed. Twenty-four hours after the administration of the yttrium, the fish were put in tanks with running sea water where they were kept until killed rapidly by flooding the gill chamber with ether. After removing the eyes and the visceral organs, the remainder of the fish was put in a pressure cooker which was brought to 20 pounds pressure and then allowed to cool. This process allows the skeleton and integument to be separated easily from the muscle with no leaching of the radioisotope. The separated organs and tissues were dried and ashed at about 550° C. The ash was spread on aluminum planchettes with the aid of a wetting agent, dried under infra-red lamps, and counted with a G-M tube and a conventional scaler for a minimum of 2560 counts. Yttrium^{91} has a maximum energy of 1.5 Mev. No corrections were made for the self-absorption which was very small at the densities employed (< 6 mg./cm.^{2}). An aliquot of the dose was counted for reference and to correct for decay.

Results

Figure 1 shows that radioyttrium is very rapidly excreted by *Tilapia.* After two days, the fishes retained only about two per cent of the ingested dose (1.6 ± 0.5). In a similar experiment, the average amount of Sr^{89} retained by *Tilapia* after two days was 20 per cent, and even after 14 days, the average retention was about six per cent (Boroughs, Townsley and Hiatt, 1956). The actual absorbed dose may be something less than these values, because marine fishes swallow water in order to maintain their osmotic balance. A small amount of recently swallowed water would therefore be trapped in the gut unabsorbed, but contributing to the radioactivity in the visceral organs. Any feces remaining in the gut would also add to the radioactivity of the visceral organs. In both instances, however, the amount would be small.

Figure 2 indicates that most of the yttrium is retained in the viscera. In rats, the liver, kidney and spleen accumulate yttrium (Hamilton, 1948), but our methods did not permit us to localize the accumulation of yttrium in the visceral organs. Future experiments to disclose this are underway. Assuming that all the radioactivity recovered in the 14-day fishes was absorbed (1.3 per cent of the dose), the viscera retained 43 per cent, the muscle 29 per cent, the skeleton 16 per cent, the integument 8 per cent, and the gills 4 per cent. The large percentage of yttrium accumulated in the fish muscle was wholly unexpected, for in rats 65 per cent of the absorbed dose was found in the bones (Hamilton, 1948).

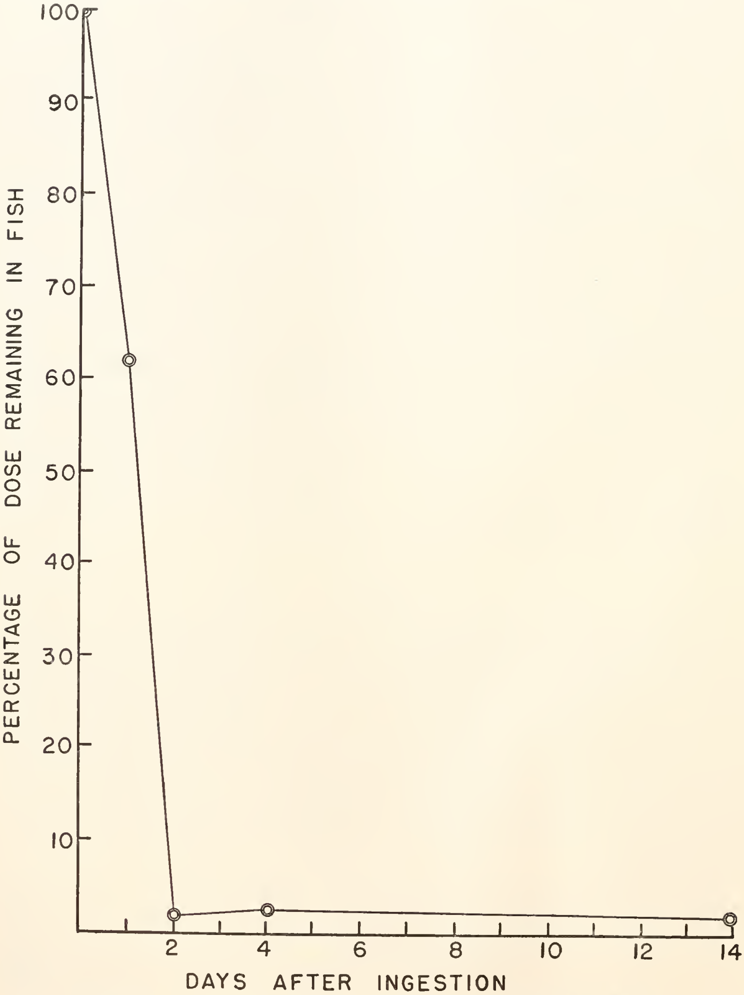

FIGURE 1. The loss of yttrium^{91} after ingestion by *Tilapia mossambica*.

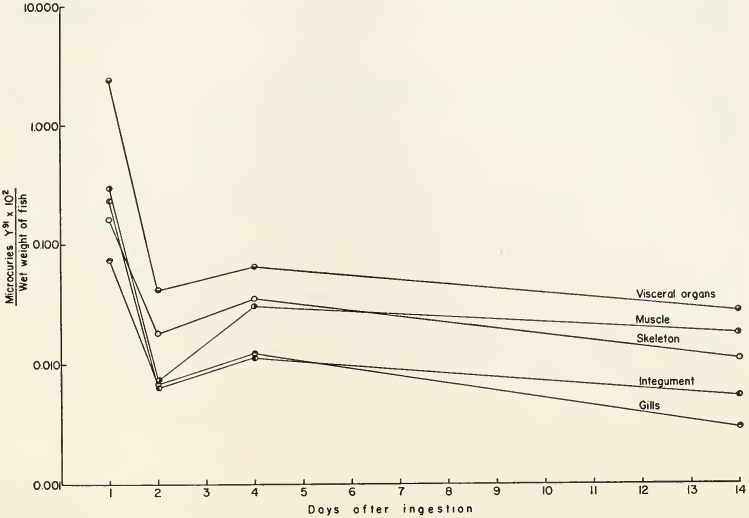

FIGURE 2. The internal distribution of yttrium^{91} fed to *Tilapia mossambica* via stomach tube.

DISCUSSION

Similar experiments with radiostrontium (Boroughs, Townsley and Hiatt, 1956) have indicated that in 14 days, *Tilapia* have about 60 per cent of the absorbed dose in the skeleton, 28 per cent in the integument, 9 per cent in the gills, 2 per cent in the muscle, and 1 per cent in the viscera. The rapid excretion of radioyttrium by *Tilapia,* coupled with the different patterns of internal distribution, indicate that the metabolism of strontium and yttrium in this species is markedly different. However, this is not too surprising in view of the difference in chemical behavior between these two elements. Studies on the accumulation of strontium by pelagic fish (Boroughs, Townsley and Hiatt, 1956) showed that this element is rapidly absorbed by all the tissues, but is also rapidly lost from the visceral organs and the blood. Similar experiments with *Tilapia,* a small sluggish fish, were unsatisfactory because of the large variability in the amount of Sr^{89} absorbed over periods up to 24 hours. We therefore made no attempt to follow the pathway of yttrium immediately after ingestion, but very likely yttrium is not actually absorbed to the extent that is strontium. At 24 hours, about 60 per cent of the dose was still in the entire fish, but most of this was in the gut. However, at least 5 per cent of the dose was absorbed by the muscle alone. This is at least 100 times more than the amount absorbed by rats from an oral dose (Hamilton, 1948), and, moreover, represents a minimum value for *Tilapia.*

The maximum percentage of ingested strontium which was recovered in the feces of *Tilapia* during 24 hours was less than 1 per cent. The feces were removed six or seven times during this interval, but some leaching of Sr^{89} may have occurred.

The percentage of Y^{91} recovered in parallel experiments has been as much as 20 per cent of the dose.

If the length of time required for an organ to excrete one-half of its concentration of a particular element is very long (biological half-life), then the effective biological half-life approaches the half-life of the radioisotope involved (radioactive decay). Elements which lodge in mammalian bones appear to have a very long biological half-life. According to Figure 2, however, the biological half-life of yttrium in all the tissues of *Tilapia* is of the order of one month. This value is of course only a guess, and long term experiments will have to be carried out to verify this estimate. In man, the body burden tolerance for Y^{91} is given as about 15 times that of Sr^{90} (N.B.S. Handbook 52), and in both instances the bulk of the radioactivity appears in the skeleton. In one year, the radioactivity owing to Y^{91} would be reduced to a negligible amount because of both excretion and decay, but very little Sr^{90} would be lost in this time. However, in organisms other than man, particularly marine organisms, one year may be a substantial part of their life span, and it is for this reason that we urge that attention be paid to the metabolism of radioactive fission products other than strontium. While strontium may constitute the most serious *direct* health hazard to man, long term effects of other shorter-lived fission products may have significant effects on the shorter-lived biota, and thus ultimately may also prove of importance to man.

There is no evidence that the slight increase in the radioactivity of the oceans has as yet caused any adverse ecological changes. Moreover, there is no evidence that adverse ecological changes will occur even as a result of the introduction of much larger amounts of radioactivity from nuclear reactor plants which are certain to be established within the next decade or so. If the present power requirements of the world are to be met with the aid of atomic energy, it is likely that a sort of steady-state condition will occur with regard to the added radioactivity in the oceans—the result of a balance between the rate of introduction of radioactive wastes, the rate of physical decay, and the rate of biological turnover. It is therefore imperative that marine biologists study in great detail the problems of the uptake and accumulation of fission products, the transfer of these nuclides back and forth among the trophic levels, and the direct, long-term effects of the nuclides in specific regions. Estuaries and the littoral zone will most likely have a higher concentration of radioactivity than the open sea, and it is in these regions that the bulk of the world's marine resources is produced.

SUMMARY

Only about 2 per cent of an ingested dose of yttrium^{91} was left in *Tilapia mossambica* after two days. This is much less than the amount of strontium retained by *Tilapia* in similar experiments. About 40 per cent of the radioisotope remaining is found in the visceral organs, but the muscles retain about 30 per cent after 14 days. The skeleton retained less than 20 per cent, the integument about 10 per cent, and the gills 5 per cent. These findings are in marked contrast with those obtained with strontium^{89} in similar experiments. Attention is focused on the fact that yttrium^{91} may have little direct effect on man compared with the possible effects of Sr^{90}, but the retention of this and other short-lived fission products in

marine organisms having a brief life span may possibly affect the biota, and thus affect man indirectly.

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IMMOBILIZING AND PRECIPITATING ANTIGENS OF PARAMECIUM

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The pattern of inheritance of the immobilization antigens of *Paramecium aurelia* and the serological basis for the specific immobilization reactions have been studied by Sonneborn (1951), Beale (1952), van Wagtendonk and van Tijn (1953) and Finger (1955). The systems of transformation from one serotype to another have also been investigated (Sonneborn, 1950a; Beale, 1948) and possible mechanisms suggested (Kimball, 1947; Sonneborn, 1950b; Delbruck, 1949). Further work on the unique systems of inheritance and stability exhibited by the immobilization antigens has been hampered by the lack of a rapid, reproducible method for assaying them. We have now been able to detect these antigens through the development of a method which involves a modification of the techniques of Oudin (1952) and Oakley and Fulthorpe (1953). Specifically, a particular band formed by the diffusion of two reactants (antigen solution and antiserum) from opposite ends of an agar column has been identified as a complex, in part, of precipitated immobilization antigen and its homologous antibody. The present paper deals with the immobilization antigens and this *in vitro* method for detecting them.

MATERIALS AND METHODS

Sera against whole animals prepared according to the methods described by Sonneborn (1950c) against several stocks of varieties 2, 4 and 8 were employed. These were tested with variety 2 antigen extracts of stock 3 and extracts of animals that were derived from the variety 2 stocks 7, 30 and 35. The survey of antigenic types found in variety 2, upon which much of the work reported here is based, has been submitted for publication. The antigen solutions were prepared in several ways: (1) Cilia were obtained from paramecia and extracted as follows (Preer and Finger, unpublished). Six million animals were concentrated by centrifugation and one volume placed in twenty volumes of 0.22% sodium chloride buffered at pH 7.8 with 0.01 *M* sodium phosphate. At the end of ten minutes the animals were centrifuged lightly at 890 *g* for two minutes; the supernatant (containing mainly cilia) was retained and again centrifuged lightly. Any sediment of animals and trichocysts formed was then removed and the process repeated until the supernatant was free of animals. The supernatant was then centrifuged for two minutes at 24,000 *g* in order to sediment the cilia. Then one ml. of 0.9% sodium chloride, buffered to pH 7.0 with 0.01 *M* sodium phosphate, was added to the centrifugate. After 24

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hours at room temperature this suspension was centrifuged at 24,000 *g* for two minutes and the clear supernatant used as antigen. (2) Lyophilized animals were placed in a 0.9% sodium chloride solution overnight in a refrigerator, centrifuged at 24,000 *g* for two minutes and the supernatant used as antigen. (3) Animals were concentrated by centrifugation, placed in a deep freeze, and, after thawing at room temperature, the entire brei was used as antigen. (4) A brei was made by repeatedly forcing living animals from a syringe against the walls of a glass cylinder at room temperature. The resulting homogenate was then centrifuged for ten minutes at 24,000 *g* and the supernatant used as antigen. These four preparations were not equally effective in yielding the immobilization antigen and are listed in increasing order of efficiency. When quantitatively comparable data were desired the antigen solutions used were, of course, obtained by the same method.

The method of gel diffusion has been given in detail by Preer (1956). It is as follows: Pyrex tubes, approximately 2-mm. inside diameter, were coated with a 0.1% agar solution, evacuated, cut into 4-cm. lengths and flame-sealed at one end. To set up a double-diffusion test 0.01 ml. of antiserum was added with a syringe to a tube held upright in a Cartesian diver loader. Then 0.6% washed, merthiolated, and buffered (pH 7.0) agar maintained at 60° C. was carefully layered with a warm syringe until an agar column of 6–8 mm. height was reached. A third layer of 0.01 ml. antigen solution was placed on the agar after it had solidified. The tube was then sealed with Picene cement and placed in a horizontal position at 24° C. When serum dilutions were used, normal serum was used as the diluent. This was to prevent mixing of antiserum with the warm agar at the time of layering. The diluent for the antigen was usually 0.9% sodium chloride or, less frequently, 10% Ringer's solution.

The location of a zone of precipitation within the agar was used as a measure of the concentration of one of the reactants (antigen or antibody) contributing to the band when the concentration of the other reactant was kept constant (Preer, 1956). The position of a band was measured after 24–72 hours with a binocular dissecting microscope provided with an eyepiece micrometer.

Variations on these general techniques, *e.g.,* mutual dilution, absorption experiments, etc. are described below.

Results

When a solution of several antigens is diffused against homologous antiserum, bands of precipitate will be formed as a result of the specific complexing of antigens with antibody. Because the position of a band is dependent on several factors (concentration of reactants, diffusion coefficients, etc.), each serologically distinct precipitating system usually appears as a separate zone of precipitation. Occasionally, however, two or more precipitating systems may, through a fortuitous combination of these factors, appear as a single band. Therefore the number of bands will represent the minimum number of antigen-antibody systems present.

Many workers have demonstrated that *P. aurelia* may manifest a number of different antigenic types, called "serotypes," and designated by the letters A, B, C, etc. When animals of a given serotype are placed into a suitable dilution of antiserum prepared against that type, their locomotion slows, and they become immobilized. Each serotype is serologically distinct from any of the others, an animal of a particular serotype generally becoming immobilized only when placed in homologous antiserum. If a number of antisera are allowed to diffuse against extracts of homologous serotypes and against extracts of heterologous serotypes, then it should be possible to correlate the presence of certain bands with these specific immobilization antigen-antibody systems.

When animals of the G serotype were used as the source of antigens and G antiserum diffused against the antigen solution, a band formed that was not present when an antigen solution prepared from non-G animals reacted with a G antiserum. Six different stocks of *P. aurelia*, all of serotype G, possessed a similar antigen, an antigen absent in extracts of two stocks of a serotype other than G and in non-G animals of two other species, *P. caudatum* and *P. polycaryum*.

Analogous results were obtained with C antigen-antiserum systems. A band appeared in these homologous systems that was missing when the antigen solution was obtained from non-C animals.

To provide further evidence that this antigen was found only in homologous serotypes, absorption experiments were performed. It was found that extracts of several stocks manifesting the G serotype were able to remove the specific precipitin from G antisera while several stocks of a different serotype and non-G *P. caudatum* and *P. polycaryum* preparations were ineffective.

The precipitating antigen associated with a particular serotype was further homologized between stocks through the use of a mutual dilution technique (Oudin, 1952; Telfer and Williams, 1953). Although all antigen preparations were able to form a distinctive band when diffused against homologous antisera, it was possible that the bands formed with different antigen preparations did not represent the same antigen-antibody systems, the correspondence being coincidental. An antigen forming a band against a particular serum may be identified with an antigen from a second extract by mutually diluting the two extracts, one with the other, and noting whether the bands formed behave independently, as though diluted with a neutral reagent, or act to reinforce each other. In order to employ the mutual dilution technique with maximum effectiveness, the concentrations of serum and antigen solution were chosen so as to eliminate most or all of the bands in the system, aside from the ones being compared, and still leave a band intense enough to withstand a two-fold dilution of antigen. For optimum resolution in a mutual dilution series the concentrations were adjusted so that the bands in the two preparations being compared formed at widely separated positions in the original undiluted systems. As a result of the mutual dilution studies, the bands formed with different preparations of the same serotype were shown to represent the same antigen-antibody system.

In summary, then, a study of serotypes G and C has shown that animals of each serotype have a specific precipitating antigen which is lacking in animals of other serotypes. It seems likely, then, that these antigens are the immobilizing antigens. Such an immobilization antigen can be identified in gel-diffusion systems in several ways: (1) a comparison of bands formed with homologous and heterologous antigens; (2) specific absorption by heterologous extracts of all antibody from a homologous antiserum, but the one that forms a zone of precipitation with homologous extracts; and (3) the mutual dilution of an extract with an antigen solution which forms several bands, one of which is known to be formed by the specific precipitinogen.

Although the immobilization antigen and the specific precipitating antigen are probably the same, there is evidence that the precipitated band may not be composed solely of immobilizing antigen and immobilizing antibody. The evidence has been obtained from a comparison of immobilization titers of antisera and precipitin titers as measured by the specific band position.

This specific band was identified as above by diffusing antisera against antigens extracted from two clones with the same genotype but of contrasting serotypes. The eleven sera titered for antibody forming this band were prepared against several different stocks and were known to immobilize animals of the G serotype in concentrations of 1 : 12.5 or less after two hours at room temperature. Eight of these anti-G sera, all having an immobilization titer of 1 : 50 or greater, precipitated a single band against the G antigen solution that was absent when preparations of the C serotype were used as reactants. Of the three anti-G sera that showed the same number and type of bands with both G and C antigen solutions, two had immobilization titers against both serotypes (F#15 and F#14B) and one (F#13B) had negligible anti-C precipitin titer. These data are presented in Table I where the immobilization titers of 24 antisera against the G and C serotypes are compared with the precipitating antibody concentration as determined by band position.

TABLE I

Comparison of immobilization titers and gel diffusion band titers of antisera against P. aurelia

Serum	Anti-G titer	G-precipitin titer	Anti-C titer	C-precipitin titer	Serum	Anti-G titer	G-precipitin titer	Anti-C titer	C-precipitin titer
P#23	100	40	0	0	F#10	0	0	100	100
P#16	100	9	0	0	P#8	0	0	100	65
P#2	75	100	0	0	F#17	0	0	50	30
P#12	75	100	0	0	F#11	0	0	40	90
P#7	50	15	0	0	F#4	0	0	25	25
P#14	50	30	0	0	F#5	0	0	25	45
P#17	50	15	0	0	F#12	0	0	20	9
F#15	20	45	15	4	P#18	0	0	15	4
F#18	15	3	0	0	F#3	0	0	15	7
F#14B	5	5	20	11	P#19	0	0	5	7
F#13B	3	1	0	1	F#1	0	0	3	0
					P#4	0	2	0	0
					P#21	0	3	0	0

The immobilization antibody titers (anti-G and anti-C titers) and the gel diffusion precipitin titers are presented on a scale with sera having the greatest concentration of antibody listed as 100, and the antibody concentration of all other sera, as compared with these sera, being denoted by numbers from 0–100. In this way, a serum with half the antibody content of the strongest sera would be given the number 50, etc. The titers presented above represent the means of several series of titrations.

Similar results were obtained with anti-C sera when the sera were titered against homologous and heterologous solutions. Ten of thirteen antisera gave a band with C antigen solutions not present when G antigen solutions were used. Of the three homologous antisera that did not precipitate this band, one had a very low immobilization titer (F#1), and two also had anti-G immobilization titer. Thus, any differences that may have existed due to the C immobilizing antibodies would be obscured (F#15 and F#14B). Seventeen sera prepared against several serotypes (omitted from Table I) and with neither G nor C immobilization antibody presented essentially identical band series with G and C antigen solutions.

Following the procedure used in the studies on antigens, absorption experiments were employed to confirm the identification of the immobilization antibody. The standard G antiserum was diffused against a G antigen extract that had previously been incubated with antiserum at 12° C. for 24 hours. Nine sera which immobilized G animals in two hours when used in dilutions of 1 : 200 or greater successfully absorbed the antigen responsible for the band found in G antigen-antibody systems. Three with poor G titers and 26 without any G titer when used as absorbents had no effect on the appearance or position of the band.

A final corroborative group of experiments was carried out using the mutual dilution method. A single serum having a high immobilization titer against G animals and precipitating antibody capable of withstanding several-fold dilution was chosen as a standard serum. When eight anti-G sera were mutually diluted with the standard serum, it was found that the antibody restricted to the G antigen-antibody reaction and present in all systems involving these sera was either related to or identical with the antibody found in the standard G antiserum, the band formed being reinforced upon the addition of the sera being tested. Additional mutual dilutions among the eight sera confirmed this finding.

Although the evidence for the identity of the band characteristic of homologous systems with the immobilization antigen and antibody is convincing, there appears to be a rather poor correlation between the titer of a serum as determined by immobilization tests and the concentration of precipitating antibody. Thus, serum P#16 with about the same G immobilization titer as sera P#23, P#2, and P#12 has less than 25% of the precipitating antibody of these antisera (Table I). Other "exceptional" sera are P#4 and P#21 which possess precipitating antibody and yet do not immobilize. It is apparent, then, that immobilization antibody and precipitating antibody, although closely correlated, are not identical. The serological nature of the relationship of the two kinds of antibodies is being studied. As would be expected, preliminary absorption experiments have demonstrated that it is possible to remove all precipitating antibody from certain antisera without abolishing all immobilizing activity.

As for the immobilization antigens, although these studies have demonstrated that in animals of serotype G one particular precipitating antigen is found and in animals of serotype C it is not found, but a second precipitating antigen is, it is not known whether immobilization antigens and precipitating antigens are identical. It is possible that only a portion of the immobilization antigen is capable of precipitation or that there may be precipitating antigen that does not take part in immobilization.

SUMMARY

1. A study of serotypes G and C, of variety 2, *Paramecium aurelia,* has been made, using diffusion in agar. It has been shown that animals of each serotype have a specific precipitating antigen which is lacking in animals of other serotypes. Consequently, it seems probable that these antigens are the immobilizing antigens.

2. Comparisons of antibody concentration, as determined by immobilization titers and by band position, show that the precipitated band may not be composed

solely of immobilizing antigen and antibody, and that there is precipitating antibody that is not capable of immobilizing.

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OOGENESIS IN HABROTROCHA TRIDENS (MILNE)

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The bdelloid rotifers of about 200 species, classified into 19 genera and 4 families, reproduce exclusively by parthenogenesis, males being unknown in this group. It is therefore interesting to study the behavior of their chromosomes during oogenesis. I have reported such a study on one of them, *Philodina roseola* (1956). Some of the findings reported in that paper are as follows: 1. In *Philodina roseola,* there are two maturation divisions, both equational. 2. No indication of synapsis has been observed between any two of the chromosomes. Individual chromosomes even in the earliest oocytes were observed to be in a condensed state. The anaphase chromosomes of the oogonial division do not despiralize in forming the nuclei found in the syncytial ovary. The chromosomes, after the last oogonial division, remain condensed, and, by progressive packing together, they form first a ring and then a homogeneous and spherical mass of chromatin occupying the center of the nucleus. When one of these nuclei is isolated by the ovary to form an oocyte, its condensed chromosomes do not despiralize into leptotene threads, but persist in a condensed state. As the germinal vesicle increases in size they separate from each other until finally 13 condensed chromosomes can be easily counted. 3. The zygoid chromosome number in this rotifer is 13. Three of the 13 chromosomes, two dot-shaped ones and one that is appreciably longer than the rest, are morphologically distinguishable from one another and from any one of the other ten (Fig. 37). It was suggested that the chromosomes in this group of animals may have lost their homology.

The present paper reports observations made on *Habrotrocha tridens,* which belongs to the family of Habrotrochidae. For methods employed in this study reference may be made to my paper dealing with *Philodina roseola.*

OBSERVATIONS

As in *Philodina roseola,* the ovary and its accessory structure, the vitellarium, in *Habrotrocha tridens* are syncytial. In mature animals, the ovary consists of about 30 nuclei, with the chromosomes in each nucleus grouped so tightly together that they form a single spherical body of smooth outline. When one of the nuclei is isolated by the ovary to form an oocyte, the individual chromosomes do not go through the meiotic changes characteristic of oocytes in other animals. They simply remove themselves from each other as contracted bodies while the nucleus increases in size.

Figure 1 illustrates the condition of the chromosomes in the nucleus of a young oocyte. At this stage, it is still difficult to differentiate the individual chromosomes. But as the nucleus enlarges, the chromosomes begin to stand out clearly as condensed bodies. If the whole history of the chromosomes in the developing egg is not studied carefully, the thread-like structures of a less basophilic character in the nuclei illustrated in Figures 2 and 3, for instance, may be erroneously interpreted as leptotene threads, and the intensely stained true chromosomes in a condensed state regarded as heterochromatic sections of thread-like chromosomes. But as the oocyte and the germinal vesicle increase in size, the true chromosomes become more and more separated from each other and more easily differentiated tinctorially from the thread-like material. The true situation can be clearly seen in Figures 2–9. The stage of maximum growth of a germinal vesicle is seen in Figure 10. At this stage the nuclear sap appears in fixed material as a fine-meshed net. Thirteen chromosomes are spread out widely apart from each other and can be most easily counted at this stage. Two of them are appreciably smaller than the others and are dot-shaped. These two, however, are not of equal size. It will be recalled that in *Philodina roseola* there are also two dot-shaped chromosomes of unequal size. But unlike *Philodina roseola,* this form does not possess among the remaining 11 chromosomes one that is conspicuously longer than the rest.

Further development from this stage is indicated by a shrinking in mass on the part of the germinal vesicle; the nucleus thus becomes reduced in size and irregular in shape (Fig. 11). But as this takes place, the nuclear sap seems to react differently to the fixative. The fine-meshed appearance no longer prevails, and threads begin to make their appearance within the nucleus (Figs. 12–16). At this stage there is a difference between the present form and *Philodina roseola.* In the latter, the nucleus keeps on decreasing in size to a much more extreme degree, and finally becomes again rounded in outline; whereas in *Habrotrocha tridens,* the nucleus stops shrinking much earlier, and I have not observed any well-rounded germinal vesicle of reduced size (Figs. 15–17). On the contrary, when the nucleus has decreased in size to the extent shown in Figure 17, it begins to break up. Figure 17a gives the condition of the same nucleus at a lower level of focus than the one at which Figure 17 was drawn. At this level of focus, the nuclear membrane shows unmistakable signs of disintegration. After the membrane is broken, the chromosomes are set free in the cytoplasm (Figs. 18 and 19). In *Philodina roseola* the chromosomes next spread out into a more or less irregular line pressed close to the wall of the oocyte. This line formation has not been observed in the present form, and Figure 19 represents the distribution of the chromosomes most frequently observed at this time of development.

At first the cytoplasm immediately surrounding the free chromosomes does not appear any different from that seen in any other area within the egg. But when the chromosomes have pulled away from the periphery of the oocyte and have become more separated from each other, they are seen to be embedded in an area of cytoplasm which appears to be more vacuolated than the rest of the cytoplasm in the developing egg (Fig. 20). This was also observed in *Philodina roseola.* Then, also as in *Philodina roseola,* it seems that under the influence of the chromosomes, a homogeneous and light-staining material is developed in which the chromosomes are embedded (Fig. 21). It is within the area occupied by this material that the spindle is developed later. There seems to be a change going on in this material, as a result of which the area formerly occupied by the homogeneous material now appears to be traversed by threads. These threads are not as taut and trim in outline as the regular spindle fibers (Fig. 22). Such stages have been frequently observed, and in some cases the fibers do give a rather close resemblance to the regular

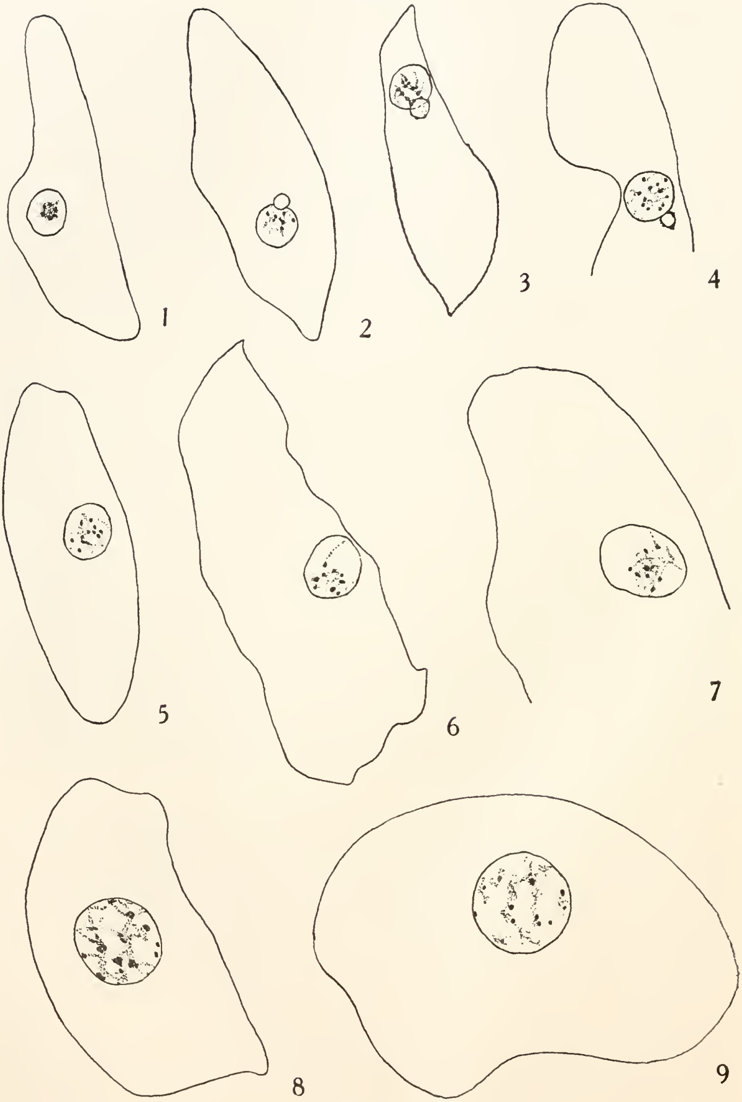

PLATE I

spindle fibers. In *Philodina roseola,* the next stage has been found to be a monopolar spindle which eventually develops into an orthrodox bipolar one (Hsu, 1956). But in *Habrotrocha tridens,* I have seen a single tripolar spindle which may be described as a compound structure formed by three different bipolar spindles, the long axes of which all lie on the same plane and so arranged with regard to each other that the structure forms a somewhat triangular configuration. The chromosomes form three separate equatorial plates, one on each component spindle (Fig. 23). Unfortunately, this is the only one I have observed in my slides.

Another spindle, also the only one I have found in my material, is represented in Figure 24. There are two cones placed at an angle as shown. The chromosomes are gathered loosely at the general area toward which the truncate ends of the two cones converge. The chromosomes are not all in one level of focus. But due to the rarity of these spindles, it is simply unsafe to consider them definitely as structures normal in *Habrotrocha tridens.* They should be merely recorded and left for future discussion when more evidence becomes available. However, in view of the peculiar spindle and its manner of development observed in *Philodina roseola* where evidence was more abundant, the possibility that the two peculiar spindles observed in *Habrotrocha tridens* may express normal stages of development in this rotifer cannot be entirely excluded. If these spindles be considered as normal structures, I should then think that they represent stages following those depicted in Figures 21 and 22 and preceding that represented by Figure 25. I would assume that Figure 24 shows a stage in which the compound spindle is breaking up and a bipolar structure is in the process of forming. This process would consist of a disintegration of the base spindle in Figure 23 and a movement on the part of the chromosomes. Then a proper rotation of the two remaining cones shown in Figure 24 would produce an orthodox bipolar spindle such as that shown in Figure 25. Of course, this is largely a conjecture.

Whatever may be the true significance of these two peculiar spindles, there is no question that a bipolar spindle does finally form to effect the first maturation division in *Habrotrocha tridens.* Figures 25, 26, 27 and 28 represent lateral and polar views of the first maturation division. In Figure 29, we see two anaphase groups of chromosomes which demonstrate beautifully that this division involves no reduction in chromosomes. Figure 30 shows a polar-body and the chromosomes within the secondary oocyte being regrouped to form the metaphase plate of the second division. Figures 31 and 32 both show the metaphase spindle of the second division, each with a polar-body directly over it at a higher level of focus (Figs. 31a and 32a), which fact indicates that the long axes of the spindles of the two divisions are perpendicular to each other. The number of chromosomes which could be made out in each one of these two metaphase spindles, counting each dumb-bell shaped granule as a unit, indicates that the second division is also equational in *Habrotrocha tridens.* Usually in such cases something like 10 to 11 units could be

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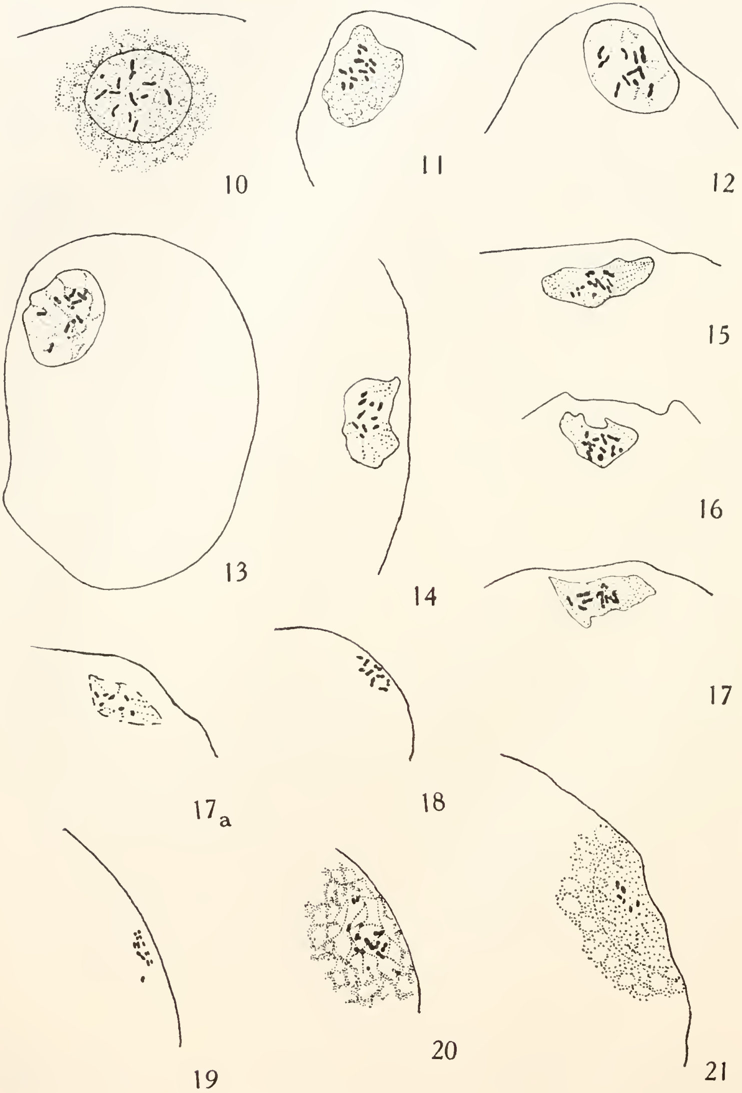
All figures are camera lucida drawings made at 1500 ×

PLATE I

FIGURES 1–9. Oocytes showing the condensed chromosomes in their germinal vesicles, and becoming progressively more easily distinguishable from the less intensely stained thread-like structures as the oocytes mature. In Figures 2, 3 and 4, an idiozome is shown in contact with the germinal vesicle.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17a
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Plate II

counted. Having seen many eggs of about this stage, I cannot help feeling that the second division takes place immediately after the first without giving the chromosomes enough time to be included within a nucleus. In *Philodina roseola,* however, a metabolic nucleus is achieved between the two divisions.

After the two maturation divisions, the comparatively large nucleus of the mature egg goes into a resting stage in which the chromosomes lose their staining intensity (Fig. 33). Figure 34 is a polar view of the metaphase plate of the first cleavage division in which 13 chromosomes with two relatively smaller ones are clearly visible. As in *Philodina roseola,* during anaphase of the first few cleavage divisions of the embryo, "elimination bodies" are visible (Fig. 35).

DISCUSSION

In both *Philodina roseola* and *Habrotrocha tridens,* the oocyte undergoes two maturation divisions, and the zygotic chromosome number, 13, is maintained in the mature egg because both these divisions are equational. No sign of chromosome pairing has been observed. I have examined as yet too few species to venture an opinion on the question as to whether or not all the species of Bdelloidea follow this pattern of oogenesis. I can only point out the fact that the two species examined belong to two different families of Bdelloidea.

In view of the genetic principles which should apply to ameiotic parthenogenetic animals, we should not be surprised to find in their chromosomes evidence of aneuploidy, polyploidy, structural rearrangement and the loss of diploid character in both the genetic and the cytological sense. In this connection I cannot do better than to quote White (1954) (p. 341): "In ameiotic parthenogenesis genetic segregation will not occur. Recessive mutations and structural rearrangements will tend to accumulate indefinitely in such organisms, only the ones which are immediately deleterious being eliminated by natural selection. Such forms must consequently be expected to become gradually more and more heterozygous, but all the offspring of a single female will resemble their mother exactly, except for newly arisen dominant mutations and differences due to the action of the environment. An ameiotic form evolving for a long period of time might be expected eventually to lose its diploid character in both the genetic and the cytological sense, its two chromosome sets having become almost completely unlike. Moreover, since no

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PLATE II

FIGURE 10. A germinal vesicle of full growth in which the thread-like structures have disappeared and a fine-meshed net has made its appearance. Note the 13 chromosomes: two dot-shaped, the rest all dumb-bell shaped indicating doubleness.

FIGURE 11. A germinal vesicle beginning to shrink, exhibiting an irregular outline.

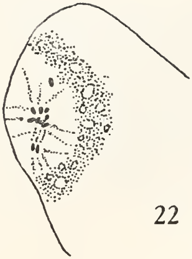
FIGURES 12–17. Germinal vesicles of increasingly reduced size in which the fine-meshed net is in turn replaced by fibers.

FIGURE 17a. The same germinal vesicle as depicted in Figure 17 but at a lower level of focus, showing signs of disintegration of its membrane.

FIGURES 18 AND 19. Chromosomes lying free in the cytoplasm close to the wall of the oocyte.

FIGURE 20. Chromosomes have pulled away from the cell wall and become more scattered in an area of material which clearly appears to be more vacuolated than the cytoplasm elsewhere in the egg.

FIGURE 21. Chromosomes lying in an area of light-staining material in which short sections of fiber can be vaguely seen.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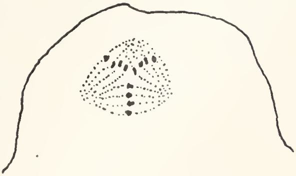
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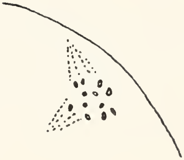
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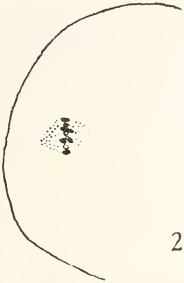
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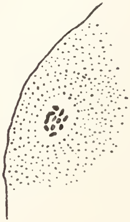
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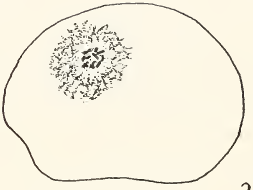
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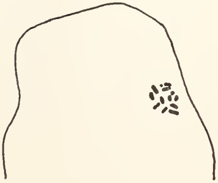
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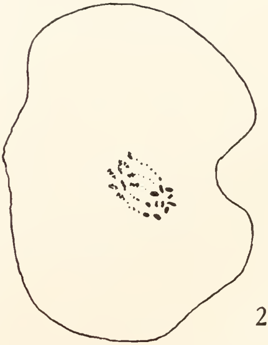
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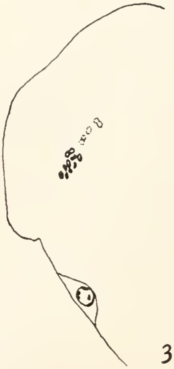
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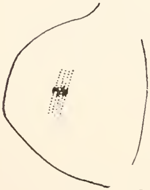
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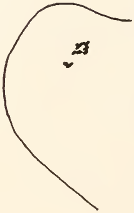
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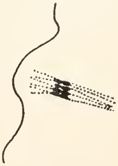
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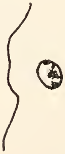
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PLATE III

pairing of chromosomes takes place during the maturation of the eggs, there is no 'mechanical' barrier to the establishment of any type of polyploidy in such forms and various forms of aneuploidy, due to irregular reduplication of some chromosome elements, must be expected to occur."

It seems to me that at least three chromosomes in *Philodina roseola* may very well have been involved in structural rearrangement of some kind, though not necessarily just among themselves (Fig. 37). This conclusion should hold unless we assume that the bisexual ancestor of this form had one pair of dimorphic chromosomes and another one without a mate. But this seems to me unlikely. Besides, although reports on chromosome number in rotifers are very confusing, none of them besides the two forms under discussion has been reported to possess an odd number of chromosomes (Makino, 1951) (p. 11). It would seem, then, that the odd number of chromosomes seen in the two species of Bdelloidea under discussion may indicate aneuploidy due either to irregular reduplication of some chromosome elements or some such structural rearrangements as centric fusion accompanied by the loss of one chromosome.

It is difficult to say whether or not the two dot-shaped chromosomes were originally members of the same homologous pair. But since they are present in both *Philodina roseola* and *Habrotrocha tridens,* and since there is no other chromosome that is comparable to them in morphology, it may be safe to look upon them as originally forming a pair. In that event, their disparity in size and the fact that the smaller one of the two, especially in *Philodina roseola,* often stains less intensely than the bigger one could perhaps be regarded as indications of loss of homology between them.

Turning next to the chromosome which in *Philodina roseola* is conspicuously longer than the rest, I must say I cannot confidently identify it in *Habrotrocha tridens.* This is the only morphological difference I can point out with confidence between the chromosome complexes of these two forms.

It should perhaps be stressed here that the absence of pairing of chromosomes in these two forms should not be interpreted necessarily as evidence of loss of homology on the part of their chromosomes, since according to the genetic principle applying to ameiotic parthenogenetic organisms the loss of the diploid character between homologous member chromosomes is possible precisely because of asynapsis.

PLATE III

FIGURE 22. Chromosomes in an area in which the light-staining material is replaced by coarse fibers reaching between the chromosomes and connecting them to the boundary of this area.

FIGURE 23. A compound spindle consisting of three bipolar spindles, each with its own equatorial chromosome plate.

FIGURE 24. A compound spindle disintegrating.

FIGURE 25. Lateral view of a first polar spindle.

FIGURES 26–28. Polar view of three equatorial plates of the first polar division.

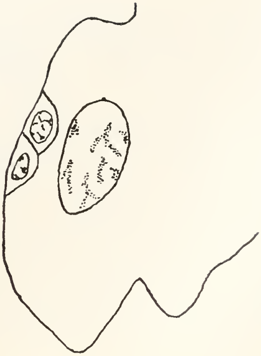
FIGURE 29. A mitotic figure at anaphase of the first maturation division.

FIGURE 30. The first polar-body and the chromosomes within the secondary oocyte regrouping to undergo the second maturation division.

FIGURE 31. A metaphase spindle of the second maturation division.

FIGURE 31a. Chromosomes belonging to the first polar-body seen at a higher level of focus directly above the metaphase spindle represented in Figure 31.

FIGURES 32–32a. The same as Figures 31 and 31a except in this case the first polar-body nucleus is already formed.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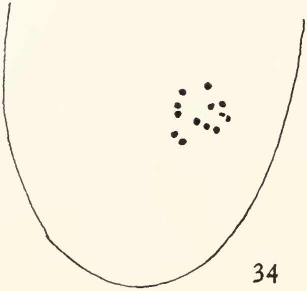
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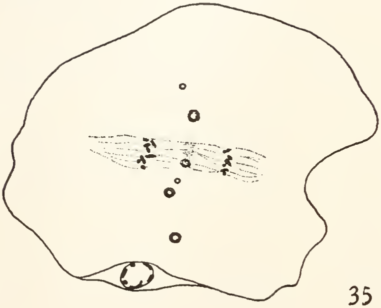
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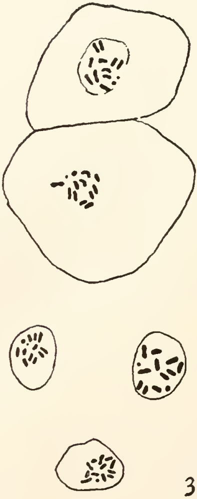
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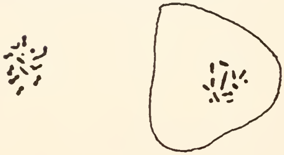
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Plate IV

In other words, asynapsis is here supposed to be antecedent to the loss of homology. Moreover, the persistent condensed state of the chromosomes in my material complicates the situation, since our current theory explaining pairing of chromosomes takes into account, besides the singleness of the threads, also the degree of their uncoiling. In this connection we should, of course, recall that in Neurospora, MacClintock (1945) has reported pairing of relatively contracted chromosomes. Incidentally, it may be mentioned that since daughter chromosomes in these forms can separate without difficulty, the coiling which their chromonemata assume must be of the paranemic type, using the term which Sparrow, Huskins and Wilson (1941) have proposed.

With regard to the first polar spindle, it must be said that due to the paucity of observations the situation in this rotifer is not clear. It is difficult to venture an opinion as to whether or not a tripolar spindle represents a normal stage of development. More observations are needed before a reliable answer can be given. At present, I can only say with confidence that the two spindles shown in Figures 23 and 24 are very distinct and unmistakable structures. Although I have not made an attempt to study the mitochondria condition in *Habrotrocha tridens,* I feel quite certain that mitochondria are not involved in this case, Devisé (1922) and Junger (1931, 1934) notwithstanding. The three separate equatorial plates of chromosomes, one on each spindle, ought to settle the question.

This research was supported by the National Science Foundation and the Washington State Initiative 171 Research Fund for Biology and Medicine. I am indebted to my colleague, Dr. W. Thomas Edmondson, for identification of the rotifer used in this investigation, and to Miss Lorraine Pilon for efficient technical assistance.

SUMMARY

1. The pattern of chromosome behavior during egg formation in *Habrotrocha tridens* is the same as that found in *Philodina roseola.* The oocytes undergo two maturation divisions, both equational.

2. The zygoid chromosome number is 13, the same as that of *Philodina roseola.*

3. The pair of dot-shaped chromosomes of unequal size is found in each of these forms, though the conspicuously longer one seen in *Philodina roseola* (Fig. 37) cannot be identified in the present form (Fig. 36).

PLATE IV

FIGURE 33. A portion of a mature egg with a comparatively large nucleus about ready to undergo the first cleavage division. Note the two polar-bodies.

FIGURE 34. Polar view of the equatorial plate of the first cleavage division. Note the 13 chromosomes, two of which are appreciably smaller than the rest.

FIGURE 35. An anaphase figure of the first cleavage division. Note the polar-body and the "elimination bodies."

FIGURE 36. One late prophase and four metaphase chromosome plates seen in the embryonic cells of *Habrotrocha tridens.* Note the two dot-shaped chromosomes.

FIGURE 37. Two metaphase chromosome plates seen in the embryonic cells of *Philodina roseola.* Note the two dot-shaped chromosomes and the one that is conspicuously longer than the rest.

4. No sign of synapsis has been observed in either form.

5. The chromosomes exist in a condensed condition in the nuclei of the ovary after the last oogonial division, and remain condensed throughout at least the first maturation division.

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SELECTIVE LIGHT ABSORPTION BY THE LENSES OF LOWER VERTEBRATES, AND ITS INFLUENCE ON SPECTRAL SENSITIVITY

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Visual processes in all vertebrates apparently depend upon a group of closely similar carotenoid-proteins. Since the spectral distribution of sensitivity is determined by the absorption spectra of these pigments, it is no accident that most vertebrates are sensitive to approximately the same band of wave-lengths. In man, this range lies between the rough limits of 400 mμ and 700 mμ. As an expression of these limitations, we have come to call wave-lengths longer than 700 mμ "infra-red" and those shorter than 400 mμ "ultra-violet."

The long-wave-length limit of sensitivity is relatively inflexible among vertebrates, because the visual pigments so far isolated from retinas do not absorb significantly above 700 mμ. At the other end of the spectrum, however, the limit imposed is of quite a different sort. The visual pigments rhodopsin, porphyropsin and iodopsin (Wald, 1955; Wald, 1939; Wald, Brown and Smith, 1955) all show considerable absorption between 300 and 400 mμ, with a secondary maximum present in this region. In the case of rhodopsin, the absorption at 360 mμ is nearly 30% of that at the 500 mμ maximum; the absorption at 600 mμ, by contrast, is less than 10%. Light of these "ultra-violet" wave-lengths is thus potentially available for utilization in the sensory process.

In the case of human vision, Wald (1952) has shown that the short-wave-length limit at approximately 400 mμ is imposed by selective absorption in ocular tissues. Below 310 mμ, in the region of general protein absorption, almost all light is absorbed by the cornea, since it is the first tissue encountered. The lens, which appears yellow in color (especially in aged persons), is an effective cut-off filter for radiations below 400 mμ. Aphakics (persons with lenses excised for cataracts) tested by Wald could read an optometrist's chart by the isolated 365 mμ line from a mercury arc, under which conditions a normal person could see nothing at all. Although the pigment responsible for the coloration of the human lens—and for its properties as a filter—has not been definitely identified, the indications are that it is a melanin (Gourevitch, 1949).

Walls and Judd (1933) and Walls (1940) attempted a comparative survey of the occurrence of such filtering lenses in other vertebrates. They found yellow lenses in the eyes of some diurnal reptiles (snakes and certain geckoes), lampreys, squirrels, tree shrews and ground squirrels (*Citellus*). None were seen in fishes or amphibians; but the yellow perch and the bowfin (*Amia*) both had yellow corneas. Walls believes that the functional advantage in the selective removal of short-wave-length radiations lies in promoting visual acuity. Chromatic aberration, since it increases exponentially as the wave-length decreases, can produce serious errors in the violet and ultra-violet (Wald and Griffin, 1947). It is therefore adaptive for diurnal animals—whose requirement is for acuity and not sensitivity—to remove this region of the spectrum before the light reaches the retina. This idea is supported by Walls' finding that yellow lenses occur only in diurnal forms.

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The mere presence of a yellow coloration, however, does not mean that the lens is a successful ultra-violet filter. Many yellow pigments (for example, xanthophyll and carotene) absorb in the blue region of the spectrum and not in the near ultra-violet. Conversely, lack of a visible yellow color does not mean that the lens is not an effective filter for the near ultra-violet. A filter absorbing nothing above 400 mμ but cutting off sharply at 390 mμ, for example, would appear colorless to the human eye, but it would be a powerful aid to visual acuity for the animal possessing it.

The fact that the human eye is a poor instrument with which to assess these properties prompted the present investigations. These experiments are an attempt to measure quantitatively the selective absorption of lenses, comparing those of a wide variety of lower vertebrates. Such measurements may clarify the functional significance of this interesting visual adaptation; in addition, they provide concrete information about an important dimension of sensory capacity in these animals. Some studies on the chemical basis of this selective absorption are also reported. Finally, the influence of these filtering lenses upon spectral sensitivity is assessed by electrophysiological measurements comparing ultra-violet sensitivity in animals with and without their lenses. A preliminary report of some of these experiments has appeared elsewhere (Milkman and Kennedy, 1955).

METHODS

For measurements of intact lens absorption, fresh lenses were dissected from the experimental animals and placed in a holder designed to fit a spectrophotometer cuvette. The holder was constructed in such a way that light passed through the lens and out an exit pupil of approximately one mm. diameter, corresponding in position to the central axis of the lens, and thence to the photocell of the spectrophotometer. Thus the absorption of only a small axial section of lens tissue was measured. The lens and holder were immersed in a cuvette filled with mineral oil, which was chosen to match as closely as possible the refractive index of the lens and thereby eliminate errors due to refraction. The mineral oil also prevented the lens from growing opaque during the period of measurement.

A Beckman DU quartz spectrophotometer was used to measure the transmission of the lenses to light of different wave-lengths. Measurements were checked repeatedly, and it was found that the transmission at a particular wave-length did not change significantly during the course of an experiment. A "blank" was used which consisted of an adapter without the lens, suspended in a similar medium. In certain species, the transmission of the cornea was measured using the same apparatus and a similar technique.

In attempts to discover the chemical basis of the selective absorption found in the lenses of certain of the species tested, aqueous extracts were made from large amounts of homogenized lens tissue. Lenses were ground with distilled water in a glass mortar; protein material was removed, either by dialysis, precipitation by heating, or making up the solutions to 50% ethanol.

Extracts were chromatographed on Whatman No. 1 filter paper in butanol-acetic acid-water mixtures (5 : 1 : 4), using ascending strips or cylinders of paper. They were viewed under ultra-violet light from a mercury arc lamp, equipped with a filter which removed almost all visible wave-lengths. The chromatograms were treated in various ways. Some were sprayed with ninhydrin; in others, the spots were cut out and eluted with a small volume of water. All absorption spectra were measured in a Beckman spectrophotometer. In some cases, it was desirable to obtain absorption spectra directly from spots on the paper; these were measured directly in the spectrophotometer, using a "blank" of dry butanol-saturated paper and employing a photo-multiplier attachment for extra sensitivity. Satisfactory spectra could be obtained in this way under conditions when eluted samples might have been too dilute to yield satisfactory measurements.

In order to measure directly the effect of selective lens absorption on spectral sensitivity, the spectral sensitivity function of intact frogs was compared with that of animals deprived of their lenses. Briefly, the technique involved recording the electroretinogram (the slow action potential of the retina) from either the cornea or, in the case of excised eyes, from the vitreous body. Moist cotton wick electrodes were used with a capacity-coupled pre-amplifier and oscilloscope. Monochromatic light produced through interference filters (or, in the case of 365 mμ, by isolation of that mercury arc line) was directed onto the eye of the preparation through a pair of opposed annular wedges which regulated the intensity. The optical system was calibrated with a thermopile and sensitive galvanometer. In each experiment, a certain amplitude of b-wave (the large positive potential of the electroretinogram) was chosen as the "criterion response." The intensity of constant-duration flash necessary to produce a response of this amplitude was then found for each wave-length, and the reciprocals of these "threshold" intensities were plotted as a spectral sensitivity curve. Frogs were curarized and dark-adapted before each experiment. Each wave-length was then tested in turn, with control flashes frequently interspersed to assure that a constant level of sensitivity was maintained. Then the lens was removed and the experiment repeated. In a number of experiments, excised eyes were used instead of intact animals; this procedure proved equally satisfactory.

RESULTS

The lenses of many of the fish and amphibians studied showed marked filtering properties. Representative absorption curves of intact lenses are shown in Figure 2, with Wald's data on the Rhesus monkey lens included for comparison. The species studied are divided into roughly three groups. Members of the first of these possess lenses which, like the human lens, show a cut-off at about 400 mμ, but they are clearly better filters in that their rise in extinction is sharper. As a result of their lack of absorption above 400 mμ, these lenses do not appear yellow. The group includes the yellow perch (*Perca flavescens*), the calico bass (*Pomoxis sparoides*), and the blue-gill sunfish (*Lepomis pallidus*), all common fresh-water species; the scup (*Stenotomus versicolor*), the summer flounder (*Paralichthys dentatus*), the rudderfish (*Serolia zonata*), and the sea robin (*Prionotus evolans*), all marine species; and the grass frog (*Rana pipiens*). The absorption spectra of lenses in this group are roughly similar (Fig. 1), except for that of the frog, in which the cut-off occurs at a definitely shorter wave-length. This will be discussed below.

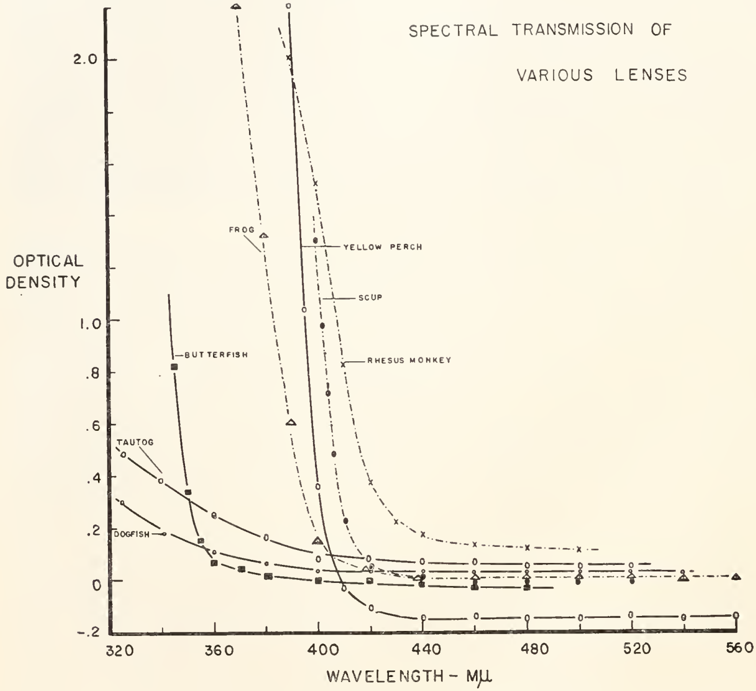

FIGURE 1. Measurements of the spectral transmission of lenses from various lower vertebrates. Data on the lens of the Rhesus monkey from Wald (1949) are included for comparison.

The butterfish (*Poronotus triacanthus*) shows instead a steep rise in lens extinction near 350 mμ, and thus is in a group by itself.

A third group, represented by the tautog (*Tautoga onitis*), the smooth dogfish (*Mustelus canis*) and the toadfish (*Opsanus tau*), all marine bottom species, and the catfish (*Amieurus nebulosus*), a fresh-water bottom scavenger, appear to have only a gradual, slight rise in lens extinction down to 320 mμ. The brook trout (*Salvelinus fontinalis*) is also in this category, but possesses a cornea which shows strong absorption beginning at 400 mμ.

Aqueous extracts of all lenses tested belonging to the first group showed absorption spectra similar to that given in Figure 2, a preparation from lenses of the cod (*Gadus callarias*). Flounder, rudderfish perch, calico bass and sea robin preparations were also tested and found to be spectrally identical; in most further studies, cod lenses were used because they could be obtained in large quantities, fresh, through the courtesy of the Booth Fisheries Corporation of Boston.

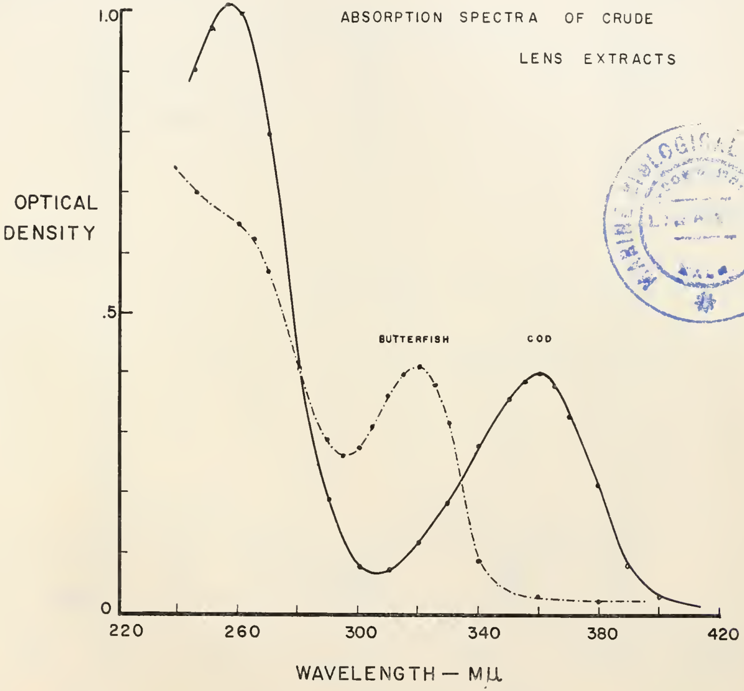

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FIGURE 2. Absorption spectra of aqueous extracts from lenses of the butterfish (dotted line) and the cod (solid line). Maxima have been adjusted to same height.

The absorption spectra of these extracts, as shown in Figure 2, show a maximum at 360 mμ, and this absorption band is responsible for the action of the lens as a cut-off filter. In the case of the frog, similar extracts had their maxima at 345 mμ, consistent with the slight displacement of the extinction of intact frog lenses.

Extracts of butterfish lenses (Fig. 2) had absorption maxima at 320 mμ, which explains the fact that intact lenses in this species have their steep rise in extinction at 350 mμ instead of near 400 mμ. No absorption bands between 300 mμ and

400 mμ were found in extracts from lenses which lacked the steep rise in extinction (third group).

The term "pigment" is usually restricted to those substances which absorb in the *human* visible range. This range, however, is restricted by the presence of an ultra-violet-absorbing lens; it is appropriate in this context to refer to the visual range of vertebrates in general as extending from 310 mμ, below which all ocular tissues absorb strongly, to 700 mμ, the upper limit of visual pigment absorption, provided no special intra-ocular filters intervene. In this sense, then, the filtering substances of the fish lens qualify as pigments, since they absorb light which is visible—though not to humans. We therefore will refer to these substances as pigments, labeling them specifically by their absorption maxima: for example, 360-

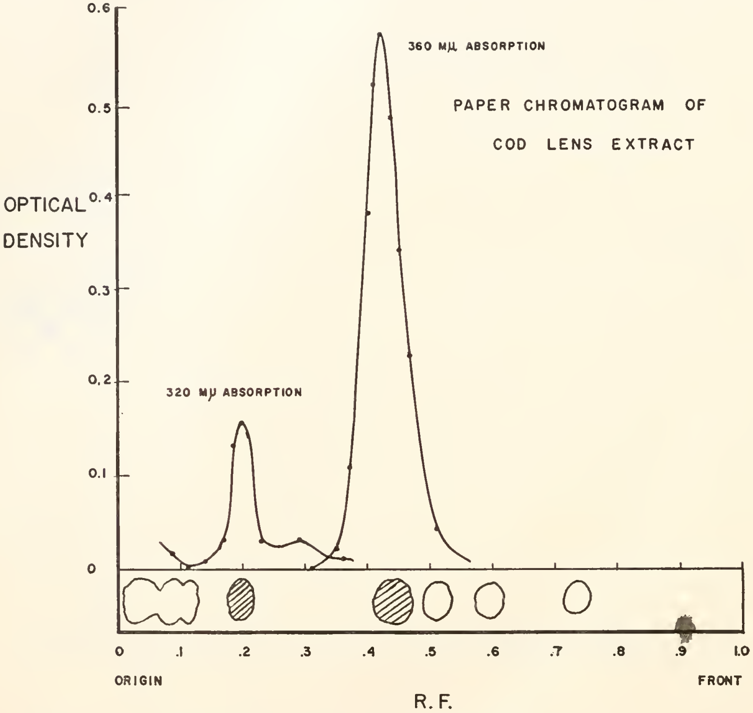

FIGURE 3. Chromatography of cod lens extract. The shaded spots at R. f. 0.19 and 0.43 are, respectively, the presumed oxidation product of 360-pigment and 360-pigment itself: the first spot corresponds with a peak of absorption measured at 320 mμ, and the second with a peak of absorption measured at 360 mμ.

pigment for the substance extracted from lenses in the first group, and 320-pigment for the material isolated from butterfish lenses.

After it was found that selective absorption by these lenses had a specific chemical basis, some attempts were made to characterize the substances responsible. Both 360-pigment and 320-pigment are water-soluble, somewhat soluble in methanol and ethanol, and insoluble in all organic solvents tried. They are stable in acid (pH 1), but in alkali (pH 12) they break down slowly and their characteristic absorption bands disappear.

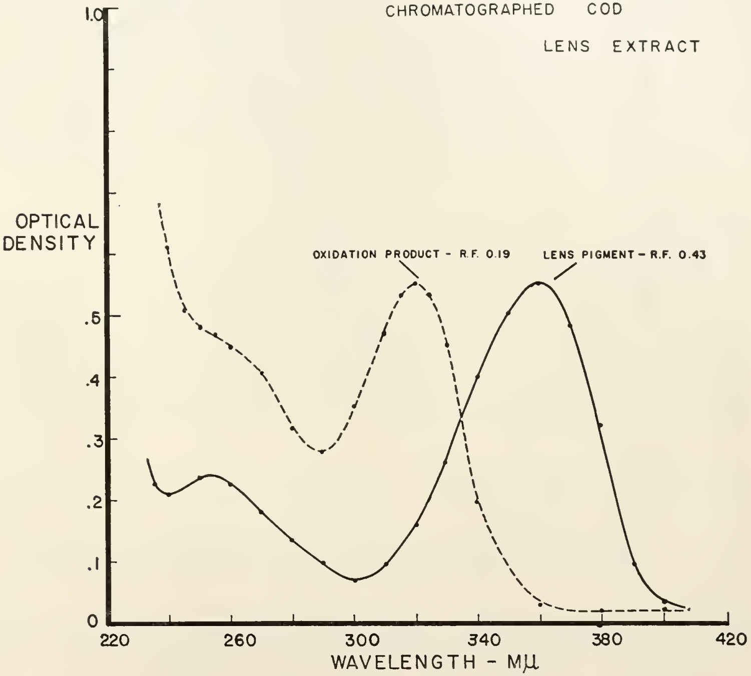

Figure 4. Absorption spectra of eluates from the two spots shown in Figure 3. Maxima have been adjusted to the same height.

360-pigment is apparently readily oxidized on standing, or by bubbling oxygen into the solution. The absorption maximum at first shifts from 360 mμ to 320 mμ; this latter band later disappears, and the final product shows only a rising general absorption into the ultra-violet, often developing a tan color suggesting the formation of a melanin-like polymer.

Paper chromatography of lens extracts in butanol-acetic acid-water mixtures (5 : 1 : 4) reveals a series of fluorescent and ninhydrin-positive spots. Presumably, a variety of amino acids and polypeptides is present, together with other substances such as riboflavin. Figure 3 shows the presence of 360-pigment as a ninhydrin-positive, non-fluorescent spot at R. f. 0.43; another spot, yellow-fluorescent and ninhydrin-positive, is present at R. f. 0.19.

Concentrated lens extracts were then chromatographed in streaks, and the bands at R. f. 0.43 were cut out and eluted with distilled water to yield a quantity of purified 360-pigment. When such eluates were allowed to stand overnight at room temperature and rechromatographed, two spots were seen: one was identical in position and absorption spectrum to the original eluted spot, and a second was present at R. f. 0.19. This spot was ninhydrin-positive and yellow-fluorescent, and had an absorption maximum at 320 mμ. This is the presumed oxidation product of 360-pigment; as can be seen from Figure 4, it is spectrally similar to 320-pigment from the butterfish.

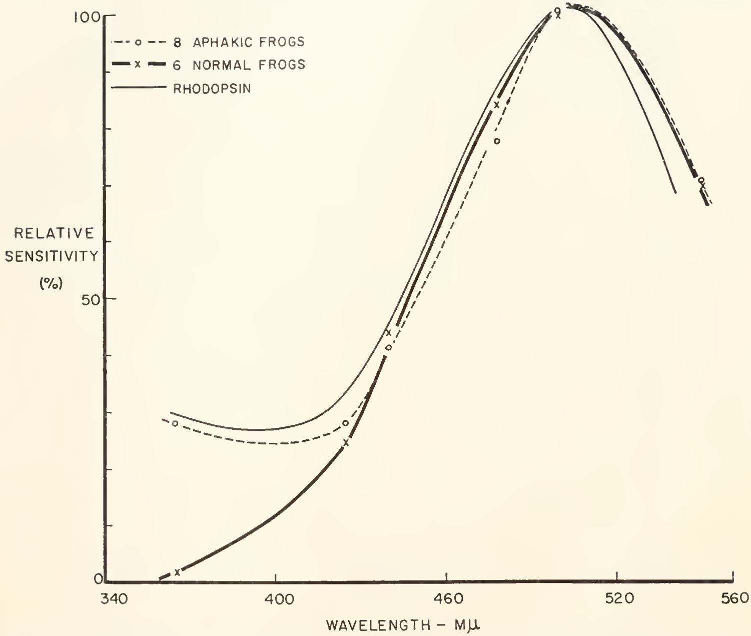

FIGURE 5. Average spectral sensitivity curves at short wave-lengths for normal frogs and frogs with lenses excised, compared with Wald's absorption spectrum for frog rhodopsin.

The ninhydrin-positive nature of both substances in these experiments indicates that an amino group is found in 360-pigment and its derivative. The absorption spectra of chromatographically purified 360-pigment and of its derivative indicate that a second absorption band at 225 mμ is characteristic of both, though in the latter it is present as a shoulder.

When iodine is added to 360-pigment in solution, a quantitative shift of the absorption maximum to 320 mμ is produced. The conversion apparently produces a single product, since there is a clear isosbestic point. It is not certain whether the observed shift is due to saturation of a conjugated double-bond system or another sort of oxidation. The product is spectrally identical with 320-pigment from the butterfish and with the previously-described derivative of 360-pigment; 320-pigment from the butterfish will not react with iodine.

The pigment from the frog lens has an absorption maximum at 345 mμ. It differs from the 360-pigment not only spectrally, but in that it will not add iodine and is acid-unstable and alkali-stable.

The effect of selective lens absorption upon the spectral sensitivity of the frog is shown in Figure 5. The spectral sensitivity function of animals with their lenses removed is in satisfactory agreement with the absorption spectrum of rhodopsin down to 365 mμ in the ultra-violet. Intact frogs, however, begin to show low sensitivity at 425 mμ, and at 365 mμ, sensitivity is only approximately 1% of that at the 500 mμ maximum.

Discussion

It appears from these results that a great many lower vertebrates, as well as mammals, possess an intra-ocular system for filtering out ultra-violet radiations which might otherwise impair visual acuity. A rough correlation is observed here, too, between the existence of such filters and an apparent ecological requirement for acute vision on the part of their possessors. The species found to lack such filters are bottom-feeders like the dogfish which rely primarily on other sensory systems in their feeding. Active, surface-living forms all seem to have filtering lenses; in the butterfish, however, the lens transmits a considerable band of ultra-violet.

The correlation is rather better among higher vertebrates. Squirrels, tree-shrews and primates, among the mammals, are largely diurnal, and have yellow lenses; no nocturnal animal has been found to possess one, and Weale (1953) has shown that the cat lens has a high transmission down to 400 mμ.

In gauging the adaptive value of an intra-ocular filtering mechanism in aquatic animals, a number of complications must be considered. First, though it is generally believed that ultra-violet light penetrates water poorly, the transmission of water for near ultra-violet (350 mμ–400 mμ) is actually quite high compared to light of 550 mμ–600 mμ (Jerlov, 1951). Second, the presence of suspended material increases scattering to a great degree. The consequences of this fact are difficult to ascertain: scattering increases exponentially with decreasing wave-length, so that the presence of suspended matter selectively increases the extinction of short-wave-lengths. However, there are some secondary considerations which cannot be ignored: a plankton-feeding fish, for example, might use ultra-violet sensitivity advantageously to locate concentrated areas of suspended matter (including organisms) by short-wave-length light scattered from them.

Walls (1942) has advanced arguments to support the idea that filtering lenses are a sort of evolutionary "second line" in the battle against chromatic aberration. Retinal cone oil droplets are held to be the usual method of filtering short-wave-length radiations. These are found in turtles and birds (yellow, red, orange and colorless), amphibians (yellow) and some fishes (colorless). Walls believes that a group which becomes nocturnal in the course of evolution loses its oil droplets; the filtering lens is evolved as a substitute in secondarily diurnal forms. This, he believes, explains the presence of yellow lenses in snakes and diurnal geckoes.

This idea does not seem to explain the situation adequately. Wald and Zussman (1938) have shown that the oil droplets of birds contain three carotenoid pigments, a different one of which is responsible for each color. The yellow one is xanthophyll, which is also responsible for the yellow coloration of the human *macula lutea.* Such droplets are unquestionably filters, but they are not filters designed for removing the ultra-violet. Xanthophyll, for example, has its absorption maximum near 450 mμ, and has declined to a very low absorption in the region of 400 mμ where chromatic aberration begins to become especially serious. The other oil-droplet pigments have their maxima at even longer wave-lengths. In the human eye, in which the cone-rich fovea is equipped with a xanthophyll filter, there is also a yellow lens.

Other evidence, too, contradicts the idea that the filtering lens is functionally identical with the oil droplets and replaces them when the latter are lost in evolution. The frog, which has been shown in these experiments to possess a sharp ultra-violet cut-off in its lens, *also* has yellow cone oil droplets. Finally, we have made observations on the yellow cornea of the yellow perch, and find that it owes its coloration to the presence of (primarily) β-carotene, a carotenoid with nearly the same absorption spectrum as xanthophyll. The perch, too, has a lens filter for ultra-violet. It thus appears that the carotenoid filters in the eyes of vertebrates (oil droplets, yellow corneas, and *macula lutea*) either serve some special function unrelated to the selective absorption of ultra-violet by the lens, or that they are accessory filters which serve to widen the band of short-wave-length absorption. In either case, they are not adaptively equivalent to an ultra-violet filter in the lens.

Evidence suggests that the pigment of the primate lens is a melanin (Gourevitch, 1949). Yellow lenses of other mammals may also owe their coloration to a melanin, although little chemical characterization has been done. The pigment can be extracted by alkali, but not by water (Walls, 1940).

The lenses of the fishes and amphibians studied here owe their selective absorption to an entirely different sort of pigment, which is water-soluble. It has not been possible so far to determine the chemical identity of these lens pigments; their behavior suggests, at least, that 360-pigment from a variety of fish and 320-pigment from the butterfish are closely related chemically.

Not many groups of water-soluble natural compounds show the type of ultraviolet spectrum exhibited by these substances. The two major groups which do are the pteridines (Forrest and Mitchell, 1954a, 1954b, 1955) and some metabolites of tryptophane such as kynurenine. There are chemical similarities between the lens pigments and these two classes of substances, but also some marked differences. At present, there is no definite basis for deciding in which group of compounds the lens pigments belong, although pteridines have been previously isolated from the eyes and integument of fish (Pirie and Simpson, 1946; Hüttel and Sprengling, 1943).

The data on frog spectral sensitivity at short wave-lengths show clearly the large effect which selective lens absorption has on actual visual processes. However, there is a quantitative discrepancy between *in vitro* measurements of lens absorption and the electrophysiological sensitivity data. Spectrophotometric measurements on the excised frog lens show that it has a transmission at 365 mμ of less than 1% of the incident light. Comparison of the relative sensitivity of normal and aphakic frogs at 365 mμ, however, reveals that the normal animals are about 5% to 10% as sensitive as those lacking lenses. The differences may be explained if it is remembered that in the spectrophotometric measurements, only a small central core of lens tissue was measured; thus, this figure represents the extinction of the longest optical path through the lens. In the intact, dark-adapted animal, with its pupil dilated, light passes through the edges of the lens as well, thus reducing its effectiveness as a filter and accounting for the difference in sensitivity.

The experiments show that the scotopic spectral sensitivity function of frogs clearly agrees with rhodopsin absorption down to 365 mμ, provided no intra-ocular filters intervene, and that an estimate of the effect of such filters in modifying spectral sensitivity may be made by measuring their transmission *in vitro*.

SUMMARY

1. Spectrophotometric studies of fresh intact lenses from a variety of fish and from frogs have shown that they are steep cut-off fibers for ultra-violet radiations, selectively absorbing almost all light of wave-length shorter than 400 mμ.

2. Certain species of fish possess lenses having high transmission in the near ultra-violet, between 320 and 400 mμ; these species must be sensitive to this spectral region, since visual pigments absorb there. Lenses of the butterfish show a steep cut-off at about 350 mμ.

3. There appears to be a correlation between possession of ultra-violet filtering lenses and a requirement for acute vision, supporting the idea that they aid visual acuity by eliminating wave-lengths which produce severe chromatic aberration. Such lenses, however, cannot be regarded as functionally equivalent to such intra-ocular carotenoid filters as retinal oil droplets and *macula lutea* since they absorb in quite different spectral regions. The theory that lens filters are an evolutionary "replacement" for oil droplets in secondarily diurnal animals is thus not in agreement with these findings.

4. Substances responsible for the properties of these lenses as filters have been extracted with water from the lens tissue. Lenses which cut off at 400 mμ yield a substance with an ultra-violet absorption maximum at 360 mμ; those of the butterfish, which cut off at 350 mμ, yield a substance with maximum absorption at 320 mμ. A presumed oxidation product of 360-pigment has been obtained which is spectrally similar to 320-pigment from the butterfish. Both substances have been characterized as to solubility, ultra-violet absorption spectra, and chromatographic behavior, but no definite identification has been made.

5. Comparison of spectral sensitivity in normal frogs and frogs deprived of their lenses has been made by recording the electroretinogram. The results show that the lens has the anticipated effect in restricting short-wave-length sensitivity. In frogs without lenses, scotopic sensitivity is in good agreement with the absorption of rhodopsin down to 360 mμ, while in normal animals sensitivity declines sharply below 400 mμ.

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RELATIVE INTENSITY OF OYSTER SETTING IN DIFFERENT YEARS IN THE SAME AREAS OF LONG ISLAND SOUND

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Quantitative studies of marine bottom invertebrates have been conducted since the early part of the century, and the results have substantially enriched our knowledge and understanding of aquatic communities. The contributions of many workers to this important branch of marine biology have been reviewed by several authors, including Spärck (1935) and, more recently, Sanders (1956).

Regardless of the progress made, there still remains one aspect of this field which has been relatively neglected but which should be of special interest to many students of bottom communities. In general, it concerns the recruitment of the new year-classes of such forms as mollusks and echinoderms that have pelagic larvae which, after a free-swimming period, descend to the bottom and metamorphose, the act commonly called setting. In particular, it deals with the variations in the intensity of setting of the same species in the same area in different years, and comparing these variations with those of other, nearby areas.

Our long-term studies of the biological events of Long Island Sound give us the opportunity to discuss certain aspects of this problem in relation to the American oyster, *Crassostrea virginica*. The conclusions are based on data collected during the past 12 years, 1944 through 1955, from ten chosen areas. The locations of these areas, which we shall call stations, are shown in Figure 1. They were confined to three depths— 10, 20 and 30 feet—and represented three major oyster-producing sections of Long Island Sound, namely, New Haven, Milford and Bridgeport. The combined area of these sections is approximately 80 square miles.

The intensity of setting at each of the stations was evaluated by counting the number of recently set oysters on special collectors consisting of wire mesh bags filled with old oyster shells (Prytherch, 1930). This is the standard method in use at our laboratory for over 20 years, and with which most oyster biologists and oystermen are now well familiar (Loosanoff and Engle, 1940; Loosanoff, Engle and Nomejko, 1955). It is important to emphasize that the locations of the stations remained the same during the 12 years, and that the methods of determining the intensity of setting were identical for all stations.

To evaluate the relative productivity of each station, we employed a simple ranking method by giving, each year, Rank 1 to the most productive station, Rank 2 to the next most productive, and so on, until the least productive was given Rank 10. For example, for 1944, Station 1, the most productive, was given Rank 1; Station 2, the least productive, Rank 10; Station 3, Rank 9; etc. (Table I).

To determine the relative productivity of the stations during the entire 12-year period, we expressed the rank of each station as the sum of its yearly ranks (Table I). Naturally, the stations that were generally better producers and, therefore, entitled to low ranks, such as Station 9, showed lower sums than the stations that were less productive. On the basis of this total score, we gave a long-range rank to each station.

The question immediately arose as to whether these ranks would remain approximately the same if the stations were graded for their performance only during the years of better sets, namely, 1944, 1945, 1946, 1953 and 1955. However, our analysis showed that the ranks of the stations for these years were not substantially different from the long-range ranks based on the 12-year observation period (Table I).

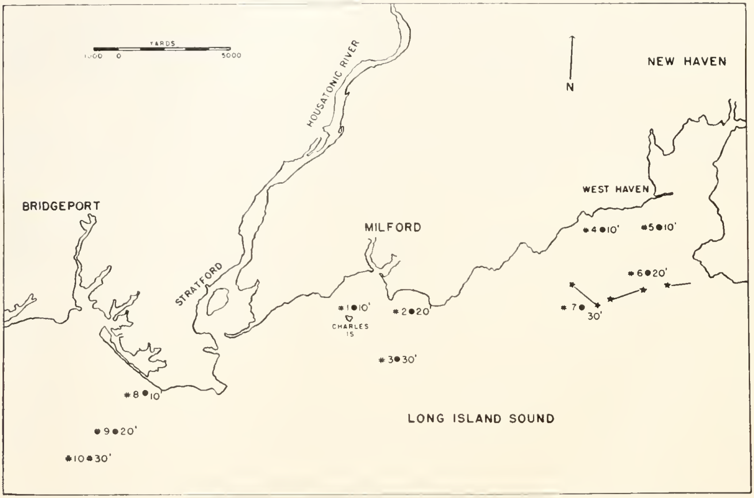

FIGURE 1. Locations and depths (in feet) of ten stations established for observation of oyster setting in Long Island Sound, 1944–1955.

A close study of the data provided information as to the relative importance of the stations, the depths, and the areas in the different years. It was established that, excluding Stations 2, 3 and 4, each station ranked first at least one year out of 12. It was also found that a 10-foot station ranked first, five times; a 20-foot station ranked first, three times; and a 30-foot station, four times. Thus, considering that at the 10-foot depth we have one station more than at 20 or 30, it appears that Rank 1 was occupied by stations of each of the three depths the same number of times.

If we add the sums of the yearly ranks of all the stations at the same depth and then calculate the average sum of the yearly ranks of these depth-groups, we will find that the 10-foot stations have a score of 65.2; 20-foot stations, 57.3; and 30-foot stations, 75.7. Thus, the 20-foot stations seem to be generally somewhat more productive than the others, while the 30-foot stations appear to be the least productive of the groups at all three depths. The latter conclusion, however, may not be well founded because the low rank of the 30-foot stations is chiefly due to the history of setting at Station 3 which, through the 12 years, was consistently one of the poorest, never rising above sixth place; whereas another 30-foot station, Number 10, was, in more than half the instances, among the five best stations, and ranked first on three occasions (Table I).

Table I

Rank crder of the sampling stations representing oyster-setting areas of Long Island Sound during the 12-year period, 1944–1955

Areas	Milford			New Haven				Bridgeport		
Stations	1	2	3	4	5	6	7	8	9	10
Depth in feet	10	20	30	10	10	20	30	10	20	30
Years										
1944	1	10	9	5	2	4	7	6	8	3
1945	1	4	8	10	6	5	9	2	3	7
1946	6	9	8	10	5	1	3	7	2	4
1947	7	6	8	9	5	2	10	1	3	4
1948	8	6.5*	10	3	6.5*	5	2	4	1	9
1949	8	4	9	3	5	7	1	2	6	10
1950	7	9	10	8	4	6	2	5	1	3
1951	9	10	7	8	4	6	5	3	2	1
1952	1	5	10	7	4	6	8	3	2	9
1953	7	3	8	10	4.5*	6	9	4.5*	2	1
1954	7	2.5*	9.5**	9.5**	1	5	6	8	4	2.5*
1955	4	5	6	10	7	9	8	3	2	1
Sum of 12 yearly ranks	66	74	102.5	92.5	54	62	70	48.5	36	54.5
Long-range rank, 12 years	6	8	10	9	3	5	7	2	1	4
Rank for 1944, 1945, 1946, 1953, 1955	3	7	9	10	5	6	8	4	2	1

*, ** Indicate a tie between stations for the same rank.

The data were also used to evaluate the relative productivity of the three areas under observation, namely, New Haven, Milford and Bridgeport (Fig. 1). By adding the ranks of all the stations, as given in Table I, for each area and year, and dividing the resulting figure by the number of stations, the average station rank for each area was determined (Table II). Accordingly, each area was given a yearly rank, the one with the lowest score occupying the first or most productive position. The sums of the average yearly ranks for each year, as shown at the bottom of Table II, were also determined. It was found that the Bridgeport area occupied first rank, or the best producing position, nine years out of 12, and never held third or last place. The New Haven area was next, occupying first rank for three years. The Milford area, however, never reached the highest position, ranked second only four times, and was in the third, or lowest position for the remaining eight years.

We cannot offer a fully satisfactory explanation for the variations or, in some instances, stability from year to year in the relative productivity of our stations. Such considerations as original number of eggs discharged; mortality of larvae due to diseases, enemies, or lack of food; and several others are, of course, of importance. Nevertheless, there is little doubt that the intensity of setting of oysters at all stations depends to a large extent upon the peculiarities of the inshore system of water currents.

Table II

Average yearly station-ranks of the New Haven, Milford and Bridgeport areas, and general rank of each of these three areas for each year of 1944–1955 period. Sums of average yearly ranks of stations of each area, and the ranking of the areas during the entire 12-year period are also given

Years	Areas			Ranks		
	New Haven	Milford	Bridgeport	1	2	3
1944	4.50	6.67	5.67	New Haven	Bridgeport	Milford
1945	7.50	4.33	4.00	Bridgeport	Milford	New Haven
1946	4.75	7.67	4.33	Bridgeport	New Haven	Milford
1947	6.50	7.00	2.67	Bridgeport	New Haven	Milford
1948	4.13	8.17	4.67	New Haven	Bridgeport	Milford
1949	4.00	7.00	6.00	New Haven	Bridgeport	Milford
1950	5.00	8.67	3.00	Bridgeport	New Haven	Milford
1951	5.75	8.67	2.00	Bridgeport	New Haven	Milford
1952	6.25	5.33	4.67	Bridgeport	Milford	New Haven
1953	7.38	6.00	2.50	Bridgeport	Milford	New Haven
1954	5.38	6.33	4.83	Bridgeport	New Haven	Milford
1955	8.50	5.00	2.00	Bridgeport	Milford	New Haven
Sum of average yearly ranks	69.64	80.84	46.34	Bpt.—9 yrs. N. H.—3 yrs. Mfd.—0 yrs.	N. H.—5 yrs. Mfd.—4 yrs. Bpt.—3 yrs.	Mfd.—8 yrs. N. H.—4 yrs. Bpt.—0 yrs.

The complexity and characteristics of these currents in the oyster-producing section of Long Island Sound are still relatively unknown because no detailed study has ever been made. We know, however, that planktotrophic larvae, with comparatively longer pelagic lives, like those of oysters and many other pelecypods, are carried by water masses and that their distribution is controlled by the currents. Under certain conditions the direction of the currents may be so changed that the larvae will be carried away from the areas where setting normally takes place, and eventually perish. In other instances, as reported by Coe (1953) for *Donax gouldi,* an enormous increase in the population of a species may occur because swarms of pelagic larvae, about ready to set, are unexpectedly brought inshore by water currents. Hence, it is understandable that the productivity of small areas, such as those designated for our stations, should, in general, be more affected by minor changes in larvae-carrying currents than that of larger areas, such as New Haven, Milford or Bridgeport, which cover many square miles of oyster-producing bottom, and should certainly display more stability in maintaining their relative positions.

These observations emphasize the importance of studying the different aspects of local minor currents, including their direction, velocity and stratifications. They also indicate the importance of understanding the relationship between the behavior of such currents and the locations of the spawning beds, of oysters or other mollusks, where the larvae originate.

Our studies suggest, moreover, that minor currents are often extremely precise in their behavior. This was well demonstrated by observations on the intensity of setting of oysters at our Station 10, located several miles from the shore and in comparatively deep water, but where, nevertheless, heavy setting continued steadily day after day for as long as three or four weeks because the currents consistently brought a supply of ready-to-set larvae to that point. In 1955, this regularity was not noticeably affected even by the strong winds of hurricane "Connie" nor by the winds and record-breaking floods of "Diane." Finally, they imply the potential danger of interfering with established combinations of the favorable ecological conditions existing on the bottom by modifying its contour so as to change the directions of the local currents. Although these changes may not affect commonly studied factors, such as temperature and salinity, they may, nevertheless, so alter the currents that the larvae will be carried to new areas, some of which may not be suitable for their setting.

We wish to express our appreciation to Barbara J. Myers for her assistance in analyzing these data, and to our colleagues, Harry C. Davis and Rita S. Riccio, for their helpful suggestions in preparing this paper.

Summary

1. During the 12 years of observations none of the stations, representing relatively small bottom areas, always occupied a position among the best oyster set producers.

2. If larger areas instead of individual stations were compared, a definite tendency of the Bridgeport area to be more productive than the others was evident.

3. There was no evidence that the stations located at a definite depth, such as 10, 20 or 30 feet, consistently produced better sets of oysters than the stations at other depths.

4. There may be a great variability in the density of oyster set even within a given depth and district in the same year. For example, Stations 4 and 5, although located in the same district and at the same depth, showed a rather different standing with Long-Range Ranks of 9 and 3, respectively.

5. Local minor water currents are important in the relative productivity of bottom areas.

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INFLUENCING THE CALLING OF SEA ROBINS (PRIONOTUS SPP.) WITH SOUND[1, 2, 3]

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Despite abundant evidence that fishes hear and produce sounds (Fish, 1948, 1954; Griffin, 1955; von Frisch, 1938), a review of the literature (Moulton and Backus, 1955) on attempts to influence fish movements with man-made sounds has uncovered reports only of quickened movements of fishes during production of such sounds. Nor has a biological significance of any sound known to stem from a fish, whether produced by stridulation of skeletal parts or by the air bladder, been clearly demonstrated. However, the apparent relationship between sound-production by some species of fishes and their respective breeding seasons has been noted by a number of authors (Fish, 1954, pp. 51, 83; Goode, 1888, p. 137; Marshall, 1954, p. 254), and the possible significance of fish calls in bringing individuals of the same species together has been suggested. Sounds are also produced during defensive spine raising of such fishes as groupers, grunts, squirrel fishes and sea robins.

During the summer of 1954, it was accidentally discovered that the production of certain fish calls, later identified as the calls of sea robins, could be stimulated by transmission of certain sounds into the water, and that the calling could be suppressed by other sounds (Moulton, 1955). The study resulting from this finding was pursued further during the summer of 1955. The observations yielded evidence that calls produced during the breeding season of two species of sea robins (*Prionotus carolinus* L. and *P. evolans* L.) are produced as responses to calls of the same species, and that by the transmission of appropriate sounds, some degree of control over the calling of sea robins may be exercised.

The sound-generating equipment employed in the experiments here described consisted of a Hewlett-Packard audio oscillator Model LAJ or a Magnecorder tape recorder Model PT6J; either an Altec Type A-323B or a Craftsman Model C550 amplifier, and a QBG transducer. The monitoring system was an AX-120 ADP or an AX-58-C Rochelle salt hydrophone and a Woods Hole Suitcase amplifier. Recordings were made on the Magnecorder tape recorder at a speed of 15 in./sec. and were analyzed on a Vibralyzer vibration analyzer. The experiments were performed from a raft anchored over 72 feet of water in Great Harbor, Woods Hole, Massachusetts.

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[2] The work was performed while the author was a Research Fellow at the Woods Hole Oceanographic Institution during the summers of 1954 and 1955.

[3] This study was supported in part by a grant from the Bowdoin College Faculty Research Fund, established by the Class of 1928.

The Sounds of Sea Robins

The sound-producing air bladder of the sea robin has been described by Fish (1954). It is the apparent source of two different calls. One of these calls is a vibrant grunt produced when a sea robin is handled in or out of water, and when a sea robin is brought to the surface by net or by hook and line. The grunt accompanies fin erection.

As determined by vibration analysis, the sea robin grunt is a single burst of noise lasting about $\frac{1}{10}$ second. The upper frequency limit is approximately 1.7 kc., the lower below 44 cps. The grunt is audible to the unaided ear above the water when a sea robin is submerged four feet beneath the surface. Noises of frequency characteristics similar to those of the grunt may be obtained by pressing the air bladder through the ventral body wall of the intact fish, and by stimulation of the nerves to the drumming muscles located on the lateral surfaces of the bilobed air bladder.

The onset of the breeding season of the sea robins at Woods Hole is marked by the production by these fishes of a staccato call. Although this call has been monitored from sea robins contained within a live car at the surface of Great Harbor, no single fish has been identified as the source of an individual call. This call is not produced under conditions that bring forth the grunt already described, and it is much more frequently produced by fishes in the Harbor than by caged specimens.

The breeding season of the sea robins at Woods Hole extends from June to September, with July and August the height of the season (Bigelow and Schroeder, 1953). In 1955, listening began on 29 June. The first staccato calls were heard on 5 July, and calls were heard on each day of listening thereafter until work terminated for the summer on 30 August. The number of outbursts of calling and the number of calls comprising a single outburst increased rapidly during the first half of July (compare Tables I and II). During the latter part of August calling became more infrequent.

Table I

Responses of sea robins to audio oscillator signals in Great Harbor, 15 July, 1955

Time	Number of signal transmissions	Response
1415	1	2 calls
1420	1	No calls
1430	1	No calls
1440	1	No calls
1450	1	No calls
1500	2	1 call after first signal
1510	3	1 call after first signal
1520	2	No calls
1530	2	No calls
1540	2	1 call after first signal
1550	3	1 call after second signal
1600	2	No calls
1610	4	1 call after signals one and two
1620	3	No calls
1630	3	No calls

TABLE II

Responses of sea robins to recordings of the staccato call and to audio oscillator signals, Great Harbor, 20 July, 1955

Time	Number and type of signal transmission	Response
1425	1 recording	2 calls
1430	1 signal	No calls
1435	1 recording	No calls
1440	1 signal	No calls
1445	1 recording	4 calls
1450	1 signal	No calls
1455	1 recording	No calls
1500	1 signal	2 calls
1505	1 recording	No calls
1510	1 signal	No calls
1515	1 recording	No calls
1520	1 signal	6 calls
1525	1 recording	No calls
1530	1 signal	No calls
1535	1 recording	No calls
1540	1 signal	No calls
1545	1 recording	No calls
(5 spontaneous calls during this interval)		
1550	1 signal	No calls
1555	1 recording	No calls
1600	1 signal	No calls
1605	1 recording	No calls
(12 spontaneous calls during this interval)		
1610	1 signal	No calls
1615	1 recording	No calls
1620	1 signal	No calls
1625	1 recording	No calls
1630	4 signals	1 call after No. 2
1635	3 recordings	No calls
1640	3 signals	No calls

The staccato calls consist of pulses of noise usually produced in pairs, at an average rate of 22 pulses/second. The paired arrangement of the pulses in a typical call is probably due to a slightly asynchronous contraction of the drumming muscles on the two lobes of the air bladder. The pairing of the pulses is not distinguishable to the ear, but can be seen on vibration analysis. Absence of the double pulses in a portion of some calls and, rarely, throughout a call suggests that one lobe of the air bladder may be silent during sound production by the other lobe.

The individual pulses of the staccato call lie between 500 cps and 4 kc. The frequencies of greatest intensity lie between 700 cps and 2.5 kc. The respective intensity peaks of the paired pulses are at different frequencies on the vibragrams, separated by approximately 1 kc. This is presumably related to a differential resonance of the two air bladder lobes which generally differ somewhat in size. It is possible to obtain sounds of similar frequency and intensity characteristics by palpitation of the dissected air bladder.

INFLUENCING PRODUCTION OF THE STACCATO CALL

With the sound-generating equipment employed, it is possible to transmit a series of sound pulses crudely imitative of the staccato call of the sea robin when the audio oscillator is set at 17 to 40 cps. (The QBG emits a considerably distorted wave train when driven with a sine wave at these frequencies.) With transmissions timed to correspond to the duration of an average call, 2½ to 3 seconds, production of the staccato call by free sea robins was repeatedly incited during July and August of 1954 and 1955.

Tables I and II present the results of two experiments extending over 2 hours and 15 minutes on 15 and 20 July, 1955. On 15 July (Table I) from one to three imitations of the staccato call were transmitted at ten-minute intervals, except for the second trial which followed the first by five minutes. Of the 15 trials, 6 were followed immediately by calling of free sea robins. There was no calling during the listening period other than immediately following signal transmissions.

On 20 July (Table II), playing of recordings of the sea robin staccato calling into the harbor water was alternated with transmission of imitations of the calling at five-minute intervals. During the 28 tests of 20 July, the calling of free sea robins was heard five times immediately following transmissions, twice after playing recordings of the calling and three times following transmissions of the imitations. Two spontaneous outbursts, frequent by 20 July, were heard during the period of the experiment. As Table II also indicates, outbursts of several calls were the rule by 20 July, whereas earlier in the month single or double calls comprised the characteristic outburst in 1955.

SUPPRESSING OF THE STACCATO CALL

Signals of 200 to 600 cps transmitted for the approximate duration of a staccato call interrupt the production of this call by sea robins. (Again the QBG signal is considerably distorted as at the lower frequency.) Signals above 2 kc. have never been effective in suppressing the calling. Signals from 600 cps to 2 kc. are variable in effectiveness. Sea robins confined in a live car and sea robins on the bottom of Great Harbor, observed by an aqualung diver, Mr. Robert Weeks of the Woods Hole Oceanographic Institution, show no obvious change in behavior during transmission of signals effective in suppressing the staccato call.

DISCUSSION

Conditions bringing forth the grunting of sea robins suggest that this sound is part of a general alarm reaction. It may be of value in nature as an adjunct to the spiny armor of the species in discouraging enemies, but no evidence is available on this point.

That the staccato calling reaches its climax near the peak of the sea robin breeding season is strongly suggestive of a relation of this calling to breeding activities, and the possibility cannot be overlooked that the calls serve as a species recognition device in waters where visibility is rather poor. Mr. Robert Weeks has informed me that visibility on the bottom of Great Harbor beneath the raft was a little over 6 feet on 10 July, 1955, and that sea robins could be seen clearly within that distance moving over the bottom of the harbor. The calling is heard at night, as well as in the daytime. In a few instances during the summer, production of the staccato call was heard to follow various sharp percussive sounds—the discharge of a high-energy spark into the water, the explosion of a detonating cap in the harbor, and the slamming of the live car lid on the raft.

Since the first staccato calls of 1955 were heard from fishes caged at the surface, while sea robins characteristically feed on the bottom, it was thought that warming of surface waters might have initiated calling from surface specimens earlier than their calling would ordinarily have commenced. However, temperatures taken with a bathythermograph during July and August of 1955 showed that there was a thorough mixing of water over the 72-foot depth under the raft, and no records obtained showed over a two-degree F. variation in temperature from the surface to the bottom.

While the significance of the calling behavior of the sea robin to its survival and normal behavior is as yet undetermined, the observations reported have demonstrated that sound is significant to the behavior of sea robins. The work has demonstrated that it is possible to control sound production by these marine fishes with man-made sounds. The findings stand as an exception to the general rule (Moulton and Backus, 1955) that production of man-made sounds causes only quickened swimming movements of fishes.

It should also be of interest to students of marine animal noises that it is possible to incite, without handling or trapping, the calling of marine fishes by transmission of appropriate signals, thus making it possible to move experiments to the natural environment from the confines of laboratory tanks, which under the best of conditions suppress and may otherwise modify calling behavior.

I am grateful for highly valued criticism to Dr. J. B. Hersey, Mr. William Schevill and Dr. R. H. Backus of the Woods Hole Oceanographic Institution, who read the manuscript of this paper. I am much indebted to Mr. Willard Dow, as to many others of the Institution, for their generous technical advice.

Summary

1. A vibrant grunt and a staccato call of sea robins in the Woods Hole area are described. Sounds similar to these can be obtained by manipulation of the air bladder and by stimulation of the nerves to the drumming muscles.

2. It is suggested that the sea robin grunt is part of a general alarm reaction, and that the staccato call is related to the breeding behavior of the sea robin. It is suggested that the staccato call may serve as a species recognition device in waters where visibility is relatively poor.

3. A method of controlling production of the staccato call is described. Production of the call can be initiated by playing into the water imitations of the call and recordings of the call itself. The calling can be suppressed by playing of signals of 200 to 600 cps, and, less consistently, by playing of signals of 600 cps to 2 kc.

4. The results obtained furnish an exception to the general rule that sound production causes only quickened swimming movements of free fishes, and demonstrate the possibility of exercising some degree of control over the behavior of fishes in nature with man-made sounds.

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CYTOLOGICAL EVIDENCE FOR A ROLE OF THE CORPUSCLES OF STANNIUS IN THE OSMOREGULATION OF TELEOSTS[1]

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Until recently there was no experimental evidence to show what tissue in teleosts was responsible for elaboration of the vital hormones of the adrenal cortex. For many years adrenal cortical function was attributed to the corpuscles of Stannius first described by Stannius in 1839. This was largely because of their morphological position on the ventral surface of the kidney, analogous to the adrenal position in other species of the vertebrate series, and because they showed histological characteristics of endocrine function. Although Giacomini (1908), in studies based on histology and morphology, attributed adrenal cortical function to secretory epithelium lining the cardinal veins, he did not relinquish the corpuscles of Stannius as a part of the adrenal complex but rather designated them as the posterior interrenal tissue. He called the glandular tissue, which is associated with the cardinal veins in the head kidney, the anterior interrenal tissue.

Many important factors have mediated against considering the corpuscles of Stannius as true adrenal tissue. Pettit (1896) demonstrated compensatory hypertrophy of one corpuscle after removal of the other in eels. However, Vincent (1898) claimed to have extirpated both corpuscles in eels without causing death to result. The inference is that if the glands were as vital in the physiology of the teleost as the adrenals are in the mammal, the eels would have been unable to survive without them. Garrett (1942) confirmed previous observations of Giacomini (1911) that the corpuscles originate embryologically from evaginations of the pronephric ducts and not from mesothelium which provides the adrenal cortical anlagen of other vertebrates. In certain forms, as *Amia,* Garrett thought that the glands might also arise from mesonephric tubules. Rasquin (1951) showed that the corpuscles were not stimulated by implantation of fresh carp pituitary or injection of mammalian ACTH as was the anterior interrenal tissue. Pickford (1953) confirmed the fact that the corpuscles were not under pituitary control by demonstrating that there was no atrophy of the glands after hypophysectomy in the marine cyprinodont *Fundulus heteroclitus,* although this investigator was also unable to find any effect of hypophysectomy on the anterior interrenal tissue. However, Chavin (1954) reported complete atrophy of anterior interrenal after hypophysectomy in the goldfish and no reaction of the corpuscles of Stannius to the same operation.

Rasquin (1951) reported that lipids were not demonstrated in anterior interrenal cells of *Astyanax* by the use of osmic acid or Sudan IV techniques. However, further investigation with more modern techniques applied to paraffin rather than frozen sections has shown that this is not the case. The use of Baker's acid

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hematein stain with acridine red, as suggested by Rennels (1953), has shown a positive reaction for phospholipids in the anterior interrenal tissue of the teleost. The diffuse nature of this tissue and the fact that patches of cells containing positive droplets alternate with those that are negative in reaction make it possible to lose positively reacting tissue in broken-up frozen sections. The discovery that the glandular cells of the corpuscles of Stannius also contained phospholipid granules provided a technique for studying the cellular reaction of the gland to various experimental procedures.

MATERIALS AND METHODS

A total of 135 individuals of the species *Astyanax mexicanus* (Filippi) were used in the course of the experiment. All were sexually mature, between one and two years of age and appeared in healthy and vigorous condition. Experimental procedures involved the injections of water, electrolytes, DCA and pitressin. Table I shows the distribution of fish among the various procedures and the times allowed to elapse between injection and killing. In each group of three or more animals, the tissues from one fish were fixed in Bouin's fluid and stained in Harris' hematoxylin and eosin; the tissues from the remaining fish in each group were fixed in calcium-formol and stained with acid hematein (Baker, 1946). The fish were killed by decapitation and the musculature from one side of the body and the air bladder were removed before placing the body in the fixing fluid. About an hour later the kidneys, containing the corpuscles of Stannius, were dissected out and returned to fresh fluid. This procedure insured rapid fixation of the rather labile granules of the glandular tissue. All tissues were imbedded in paraffin and sectioned at five microns and some were counterstained with acridine red.

The volume of all fluid injections was 0.05 cc. except for those of pitressin-and-water, where 0.15 cc. was used and the injections were made into the abdominal cavity. Glass-distilled water was used, alone and for dissolving sodium and potassium chloride. The implantation of dry DCA pellets was also made intraperitoneally. These contained 75 mg. each, and, inasmuch as this amount was far too great for the small fish, the pellets were broken up and small pieces were used. With this method there is no way of measuring the amount of hormone absorbed by any one fish. However, pieces of pellet were observed in all implanted fish at the time of death, indicating a continuous supply of hormone throughout the experimental period.

Two series of injections were made with pitressin for a study of the reaction to antidiuretic hormone. The first of these consisted of one pressor unit in 0.05 cc. aqueous solution in each fish. The second series consisted of the same amount of hormone diluted to 0.15 cc. with glass-distilled water, for the purpose of giving an additional stimulus of water load in the fish.

Weights of *Astyanax* of this age group range between one and two and one-half grams. Weighing the fish either before or after killing was avoided, first because prompt fixation was necessary, and secondly, because the fright caused by extra handling might have had some effect on granulation in the cells to be studied.

In addition, one *Astyanax* was used for each of the following methods: the pyridine extraction test (Baker, 1946) to verify the phospholipid content of the tissues reacting positively to acid hematein, Cowdry's modification of Bensley's

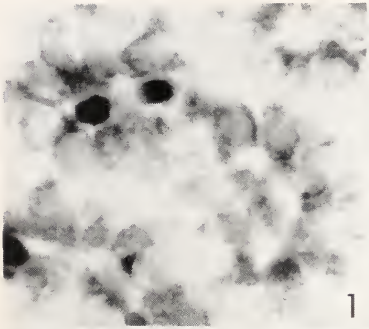
TABLE 1

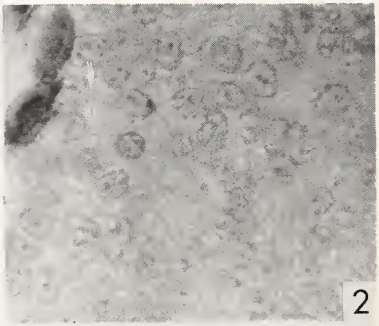
Numbers of Astyanax used and duration of experimental procedures

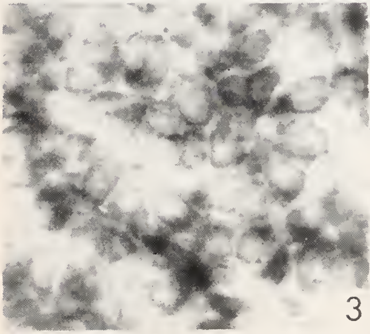
Experimental procedure	Nos. of fish	No. of days before sacrifice
Implantation of DCA pellets	3	1
	3	3
	3	5
	3	8
	3	10
	3	18
	3	25
	6	75
Daily injections 0.05 cc. distilled water	3	1
	3	3
	3	5
	4	7
Daily injections 2.0 mg. sodium chloride	3	1
	3	3
	3	5
	3	7
Daily injections 0.5 mg. potassium chloride	3	1
	3	3
	3	5
	3	7
Single injection 1.0 mg. potassium chloride	3	30 minutes
	3	1 hour
	1	2 hours
	4	24 hours
Single injection aqueous pitressin 1 unit	3	30 minutes
	3	1 hour
	3	2 hours
	3	4 hours
	3	6 hours
	3	24 hours
Single injection 1 unit pitressin plus 0.1 cc. water	3	30 minutes
	3	1 hour
	3	2 hours
	3	4 hours
	3	6 hours
	3	24 hours
Tests for pyridin extraction, mitochondria, and ascorbic acid	4	

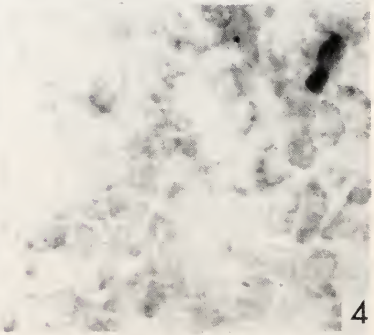
method for mitochondria as given by Jones (1950) to ascertain the nature of the granules in the cells of the corpuscles of Stannius, and Bourne's (1936) method to discover the presence or absence of ascorbic acid in the same glands.

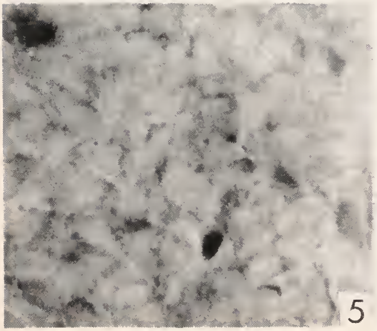
Lastly, ten fish were injected with 0.1 cc. distilled water five days a week for four weeks and ten others were allowed to live in 1% sodium chloride for ten days. The corpuscles of Stannius of all these were studied after staining with Baker's acid hematein.

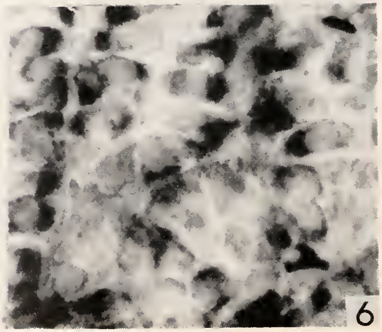












Cells of the corpuscles of Stannius of *Astyanax mexicanus* stained with Baker's acid hematein after various experimental procedures. Magnification 1200 ×.

FIGURE 1. Normal untreated fish showing blackened granules in the cytoplasm.

FIGURE 2. Cells unstained after pyridin extraction test, indicating the blackened granules to be composed of phospholipid.

Experimental Results

Many teleost tissues reacted positively to Baker's acid hematein stain: red blood cells, myelin sheaths of nerves, zymogen granules in exocrine pancreas, granules in cells of both anterior interrenal and corpuscles of Stannius, and granules of the coarse granular eosinophiles found in the connective tissues and sometimes in the blood of teleosts. The only tissues that remained positive after pyridin extraction were the erythrocytes and some of the large granules in the anterior interrenal cells.

Figure 1 is a photomicrograph showing the positive reaction to Baker's acid hematein stain in the cells of the corpuscle of Stannius of a normal, untreated *Astyanax*. The black material is made up of phospholipid granules and possibly also mitochondria. Figure 2 shows the corpuscle cells devoid of any stained granulation after application of the pyridin extraction test; the dark stained objects are erythrocytes. The corpuscle is normally made up of small granular cells that are greater both in amount of cytoplasm and size of nucleus at the periphery than at the center of the gland. Sometimes the gland has a cord-like appearance caused by two lines of cells on either side of a capillary. The nuclei are distal to the blood vessel, and the cytoplasmic granules crowded into the part of the cell adjacent to the capillary wall. At other times, probably associated with less activity, no cord-like or acinar arrangement can be detected and the cells appear to be crowded within the confines of the connective tissue capsule without any obvious architecture. Bobin (1949), using Sudan Black B and osmic acid, has also demonstrated the lipid nature of the cellular granules of the corpuscles in the European eel. In this species, she was able to distinguish both mitochondria, which were rod-like or slightly filamentous, and secretory granules, which were spherical. A similar distinction was not apparent in *Astyanax*. When stained for mitochondria, the cells were found to be crowded with these organelles which were spherical and smaller than the granules stained with acid hematein. The size difference, however, may be an artifact related to the different fixing and staining process. The probability is that acid hematein stains both types of inclusions at the same time. After the corpuscle cells are degranulated by experimental procedures there is a simultaneous loss of so much cytoplasm that mere non-reactivity of mitochondria cannot be responsible for the loss of staining reaction. The application of acid silver nitrate for demonstration of ascorbic acid resulted in only very rare stained granules in occasional corpuscle cells. However, Fontaine and Hatey (1955) have found a high content of ascorbic acid in these glands in the salmon.

Effects of desoxycorticosterone acetate (DCA)

Implantation of DCA pellets brought about an enlargement of the cells of the corpuscles with a simultaneous increase in number and size of cytoplasmic granules.

FIGURE 3. Increase in granulation in corpuscle cells of a fish that had received injections of water five days a week for four weeks.

FIGURE 4. Decrease in granulation in corpuscle cells of a fish that had lived in 1% saline for ten days.

FIGURE 5. Decrease in granulation 6 hours after injection of one unit undiluted aqueous pitressin.

FIGURE 6. Increase in granulation 6 hours after injection of one unit pitressin plus an added water load.

The hypertrophy of the cells with their heavy granulation, particularly at the periphery of the glands, was observed as early as three days after implantation. After 18 days, heavy granulation was seen in all the cells throughout the gland. At the same time, the cord-like arrangement of the cellular elements along the capillaries was pronounced, particularly noticeable under the low power of the microscope. This reaction was maintained throughout the 75-day period. The hypophyses of the three animals killed 18 days after implantation were sectioned and stained with Masson's trichrome stain. Histological study revealed that these glands were apparently normal in every detail. Prolonged administration of DCA had no such effect on the transitional lobes as was observed by Rasquin and Atz (1952) after injection of cortisone in the same species. Administration of cortisone brought about an inversion of the ratio of acidophils to basophils with subsequent marked acidophilia of the lobe.

Effects of water

The same results in the cells of the corpuscles of Stannius, enlargement and heavy granulation, were obtained by injections of distilled water. However, study of the glands on the first and third days after injections were started showed an initial shrinkage of the cells, causing spaces to occur between them, and there was some evidence of degranulation on the first day. From the fifth day onward the cells were hypertrophied and heavily granulated. The granulations were evident even in the hematoxylin and eosin-stained sections where they were markedly acidophilic. Figure 3 is a photomicrograph of the corpuscle of a fish injected five days a week for four weeks with distilled water. Heavy granulation is very evident here. Furthermore, hypertrophy of the entire gland was seen in most of the ten fish subjected to this procedure; sometimes the hypertrophy occurred in only one corpuscle so that the hypertrophied organ would be twice the size of the other one in the same animal.

Effects of sodium chloride

Continued sodium chloride injection at a dosage of 2 mg. per day brought about only slight hypertrophy of the cells of the corpuscles of Stannius and granulation appeared about the same as that seen in normal glands. However, the glands in the fish that lived 10 days in 1% saline showed degranulation of the cells. This reaction is seen in Figure 4.

Effects of potassium chloride

Because of the toxicity of potassium chloride the daily dose had to be reduced to 0.5 mg. in order to ensure survival. Doses of one mg. each were fatal, the fish dying between two and 24 hours after injection. Some of these were preserved for study (Table I). After one injection of 0.5 mg., the cells of the Stannius corpuscles appeared large and heavily granulated. Subsequently degranulation occurred and the cells were much smaller in size. In addition, the cord-like arrangement of the cells was disrupted and red blood cells were scarce as a result of decreased blood supply. Degranulation was obvious in all fish dead 24 hours after the one-mg. dose. In sections stained with hematoxylin and eosin, it could be plainly seen that the degranulation resulted in considerable loss of cytoplasm from the cells. Nuclei were crowded together, especially in the center of the gland where they were virtually denuded of cytoplasm. In the corpuscles of the fish receiving the smaller daily doses complete degranulation was not seen; some glands contained more stained granules than others but in general, all the cells were smaller than normal and the granulation was fine and usually confined to a small area about the nucleus.

Effects of pitressin

The cells of the corpuscles of Stannius reacted differently to the two procedures employed for pitressin administration. With pitressin alone, the cells were degranulated and decreased in size although this was not so extreme as when potassium chloride was used. One-half hour after injection, the cells showed a very fine granulation distributed mostly in a narrow ring around the nucleus. The same picture was obtained after one hour except that the hematoxylin and eosin-stained sections showed the nuclei to be somewhat shrunken and hyperchromatic. After two hours the granules seemed larger and more numerous and this slightly heavier granulation persisted up to six hours after injection. By 24 hours, however, the gland had returned to its normal appearance. Figure 5 represents the corpuscle cells six hours after injection of pitressin.

In great contrast to Figure 5 is Figure 6 which represents the corpuscle cells six hours after the injection of diluted pitressin. The hypertrophied cells with heavy black granulation were typical of all the corpuscles from one to six hours after injection. After only one-half hour the cells appeared small and granulation was fine and confined mainly to a ring around the nucleus, as described for the injections of pitressin alone. After 24 hours, the corpuscle, although still heavily granulated, had begun to take on a more normal, lighter stained appearance.

All the experimental procedures, with the exception of pitressin injections, served to decrease the staining response of mitochondria in kidney tubules. In the case of DCA administration, the staining reactivity returned to the mitochondria of the tubules after 75 days, indicating that the fish had made some physiological adjustment to long continued administration of this hormone. The kidney tubules of all fish included in the pitressin-injected group showed deeply stained mitochondria, especially noticeable in the more distal parts of the tubules, the intermediate segments and the ureters.

Discussion

Much of the literature pertaining to the corpuscles of Stannius is now of historical interest only. A full bibliography up to 1946 was published by Aboim. The most recent contribution is by Bauchot (1953) who studied the comparative anatomy of the glands in 47 different species including both marine and fresh water forms, attempting to relate their anatomical location to phylogeny. He concluded that the most primitive position of the corpuscles is an anterior one about midway of the length of the kidney, and the most evolved, a posterior one, much nearer the vent, although there were exceptions, as in the salmonids and *Solea* where the location of the corpuscles was not compatible with the systematic position of the fishes. This author also considered the number of corpuscles to have a phylogenetic significance. Thus the holostean, *Amia,* possesses between 40 and 50 corpuscles and the salmonids anywhere from six to 14, while the usual number for most teleosts is two. In *Astyanax* the number was found to vary between two and four, although two was by far the most common. Garrett (1942) also thought the large number of corpuscles was a more primitive condition, the advanced condition of two major corpuscles being produced by the fusion of many smaller ones. Garrett, after demonstrating the origin of the corpuscles from the pronephric duct, suggested a homology of the glands with a part of the Mullerian duct and Bauchot is in agreement with this suggestion. Some of the reasoning behind this idea is concerned with the fact that the chondrosteans, in which the corpuscles of Stannius are absent, have reduced and non-functional Mullerian ducts, while the holosteans and teleosteans, in which there are remnants of the Mullerian ducts, possess the corpuscles of Stannius.

In general, two kinds of changes were brought about in the cells of the corpuscles by the experimental procedures. Degranulation, loss of cytoplasm and consequent decrease in size of cells accompanied the administration of potassium chloride, undiluted pitressin, and long-continued immersion in saline. Hypertrophy of cells with increase in numbers and size of blackened granules accompanied the administration of water, diluted pitressin and DCA. The non-reactivity of the cells after sodium chloride injection may be owing to the fact that the dosage was too small to have an effect. Unfortunately, little is known about the action of DCA in fish.

Final interpretation of these results must await further study, particularly by investigators who have physiological techniques at their disposal. It is possible that the corpuscles were responding merely to the increased water load, that degranulation after administration of potassium chloride was owing to the toxic effects of the potassium ion and that the degranulation after undiluted pitressin was an initial release of secretion unaccompanied by further immediate stimulation. It seems fairly obvious from these results that the corpuscles of Stannius have some function in osmoregulation, inasmuch as changes in the granulation are accompanied by changes in the metabolic activity of the kidney tubules.

If the function of the corpuscles has to do with water excretion it might help to explain why various investigators have been unable to demonstrate water retention in teleosts after administration of posterior lobe hormones. Burgess, Harvey and Marshall (1933) were unable to demonstrate any effect on urine flow in the catfish, *Ameiurus nebulosus,* with 0.2 to 2.0 units of pitressin per kilogram. Their graph shows a slight increase in water diuresis for the catfish after pitressin injection, probably without statistical significance. Boyd and Dingwall (1939), using pituitrin, were unable to cause an increase in weight in young carp, although comparable doses of the hormone acted positively on frogs to increase the weight as a consequence of water retention. Fontaine and Raffy (1950) thought that the failure might have been due to the use of mammalian hormone and therefore they repeated the experiments with preparations made from the pituitaries of fish, carp, eels, etc. Their fish posterior pituitary preparations proved to be potent in causing water retention in frogs, but negative results were still obtained in the fish.

Callamand *et al.* (1951) reported that the hypophysis was not concerned with osmoregulation in eels inasmuch as they were able to place hypophysectomized

Anguilla back and forth from fresh water to sea water and even into water with twice the salinity of sea water without any deleterious effects. On the other hand, Pickford (1953) found that hypophysectomized *Fundulus heteroclitus* were unable to survive in fresh water or diluted sea water, although this species is normally euryhaline. Burden (1956) was able to keep these fish alive in fresh water by replacement therapy of *Fundulus* pituitary material. He postulated the secretion of an unknown factor by the *Fundulus* pituitary which regulates the salt balance of the fish in fresh water. Other investigators (Matthews, 1933; Abramowitz, 1937) have reported no difficulty maintaining hypophysectomized *Fundulus* in fresh water.

Neurosecretory material in the hypophysis and hypothalamus of a teleost was first described by Scharrer (1932). Since then Arvy, Fontaine and Gabe (1954) have shown that neurosecretory material in the hypothalamo-hypophyseal systems of *Phoxinus* and *Anguilla* can be depleted by subjecting the fish to hypertonic solutions, indicating a sensitivity of the neurosecretory apparatus to the need for retaining water in the internal environment. Rasquin and Stoll (1955) have shown that neurosecretion may be withheld in the brain nuclei after injection of pitressin, indicating a reaction to the antidiuretic principle, even though it has not yet been demonstrated physiologically.

Interpretations of cellular activity in the corpuscles of Stannius for this report depend mainly on the reaction of the cells to Baker's acid hematein stain for phospholipids. Unfortunately the significance of phospholipin in cellular metabolism is not yet thoroughly understood. Among several theories reviewed by Sinclair (1934), one considers that phospholipids are increased during cellular activity, particularly in actively secreting glands such as the salivary glands and the corpus luteum. Rennels (1953) also believes that phospholipids play an important role in the secretory activity, citing the staining reaction of hypophyseal acidophils, adrenal cortical cells and mitochondria. He points out that different phases of activity of both secretory granules and mitochondria are accompanied by positive or negative reactions to the stain. After gonadectomy, mitochondria of the delta cells of the rat hypophysis showed an increased activity presumably associated with increased secretory function of the cells, even though the secretory granules of these cells have no affinity for the stain.

Cain and Harrison (1950) have also suggested that histochemically demonstrable phospholipid is connected with some special metabolic activity. In a cytological study of the adrenal cortical cells in the rat, they have shown that mitochondria have an affinity for acid hematein during the phase of active secretion in the cell, and that after discharge of secretory products, the mitochondria become negative to the stain. The mitochondria become positive to the stain before the lipid droplets, but the droplets are not formed from the mitochondria; rather they are separate and distinct within the cytoplasm. Therefore, for the present report, the increase in positive staining response of the corpuscles of Stannius has been interpreted as an indication of increased metabolic activity. This interpretation is strengthened by simultaneous hypertrophy of the cells and of the whole organ with increased stainable granulation.

All these results strongly indicate the presence of a special mechanism antagonistic to the antidiuretic hormone in teleosts, and this may possibly be produced by the corpuscles of Stannius which are not found in other vertebrates.

SUMMARY

1. The effects of DCA, pitressin, water, and sodium and potassium chloride on the cytology of the corpuscles of Stannius were studied by means of Baker's acid hematein stain for phospholipids. The fresh water characin *Astyanax mexicanus* was used.

2. Two kinds of changes in the cells of the corpuscles were brought about by the experimental procedures: degranulation, loss of cytoplasm and consequent decrease in size of cells accompanied the administration of potassium chloride, undiluted pitressin and long continued immersion in 1% sodium chloride, and hypertrophy of cells with increase in numbers and size of blackened granules accompanied the administration of water, diluted pitressin and DCA.

3. Loss of staining reaction in mitochondria of kidney tubules was associated with increased secretory activity in the corpuscles of Stannius except in the case of long continued DCA administration and administration of pitressin.

4. The results are interpreted as indicating a function of the corpuscles of Stannius in the osmoregulation of these fish possibly connected with excretion of excess water.

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ON THE ECOLOGY OF THE LOWER MARINE FUNGI [1, 2]

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The lower marine fungi (*i.e.,* Myxomycetes and aquatic Phycomycetes) have generally been described as occurring on plant and animal hosts. While several forms have been described as saprophytes, the only genus known to occur on debris is *Labyrinthula* (Sparrow, 1936). All other described species are endobiotic or epibiotic with rhizoids penetrating living or dead host cells. They are also sporadic in occurrence. The application of a semi-quantitative plating technique to sea water has now established that lower fungi are far more common in littoral waters than previous studies indicate, and has suggested a new ecological niche for these fungi.

The plating technique which we used consisted of spreading samples of sea water with a bent glass rod on the surface of a solid isolation medium (Table I).

TABLE I

Isolation Medium

Sea water	80 ml./100 ml.
Gelatin hydrolysate	0.1%
Glucose (added aseptically)	0.1%
Liver 1:20	0.001%
B-vitamins	
Agar	1.5%
	adjusted to pH 7.5

Marine mineral base (Vishniac, 1955) was sometimes substituted for sea water. Gelatin hydrolysate and the B-vitamin mixture were prepared as by Vishniac and Watson (1953). Liver extract concentrate 1:20 was obtained from the Nutritional Biochemicals Co.

The moisture content of the medium is critical: the agar plates should be dried overnight, but not allowed to stand for more than two days. Just before use the plates are spread with 2000 units of Penicillin G (Squibb, buffered) and 0.5 mg. of dihydrostreptomycin sulfate (Wyeth) in concentrated aqueous solution. These plates will then absorb a 0.2-ml. sea water sample in an hour or two. After inoculation, the plates were incubated at 20 degrees or less. Such plates support the growth of lower fungi, yeasts, molds, and some diatoms, but few or no bacteria. We have found unfortunately incomplete suppression of bacterial growth when water samples taken from City Point, New Haven, near the sewage disposal plant,

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were plated. Colonies of lower fungi visible to the naked eye appear in a week or ten days. Colonies were counted at 30 × and further examined at 100 ×.

The results of spreading triplicate 0.2-ml. samples of sea water taken in June, 1956 from waters in and around Woods Hole, Mass. are given in Table II. The number of species represented is probably a minimum figure, since only colonies which were markedly distinct in color, texture, or size of thallus were picked for further study. Yeasts, molds and diatoms were not counted regularly. Yeasts were rare. From 0–5 colonies of molds, mainly *Penicillium* spp. of uncertain provenance, were found on those plates for which molds were counted. The presence of molds was not correlated with the presence of lower fungi.

It is evident from the poor agreement between triplicate platings in Table II that this technique is quantitative only within an order of magnitude. The procedure suffers from the following defects:

TABLE II

Occurrence of lower fungi in sea water

Origin of sample	Colonies/plate	No. species
1. Sea water tap, W.H.O.I.	0, 0, 0	0
2. Great Harbor	0, 0, 0	0
3. Eel Pond	94, 34, 20	7
Tap water, from bowls with algae		
4. Algae washed ca. 5 hrs.	23, 8, 20	5
5. Algae washed ca. 24 hrs. (another collection)	15, 10, 1	6
Water expressed from algae		
6. Pilings, U. S. Fish and Wildlife Station	62, 5, 56	2
7. Rocks, Red Spindle (Grassy Island)	66, 214, 35	7

(1) Spreading the sample, necessitated by the aerobic nature and poor temperature tolerance of the desired forms, entails the loss of 3 to 8% of a 0.2-ml. sample. The amount of sample remaining on the glass rod was determined by weighing the salt remaining on the glass rod after spreading a 20% NaCl solution.

(2) Either a thallus or a spore may give rise to a colony. The ecologic implications of the presence of a thallus or a spore in the sample are quite different.

(3) A thallus may produce zoospores in the interval between spreading and drying of the sample, giving rise to several colonies. The occasional appearance of groups of colonies of the same form on a plate was presumed to have this cause.

(4) Not every viable spore or thallus present in the sample may give rise to a colony. The conditions provided for growth were chosen after study of a limited number of marine forms—members of the genera *Labyrinthula, Sirolpidium, Thraustochytrium,* and three unidentified isolates. Obviously, forms with other requirements may exist. We have been particularly interested in assessing the probable extent of the selective action of the medium.

It is probable that forms with additional nutritional requirements would grow sufficiently on the medium used to form a countable colony. The ability of an individual organism, previously well nourished, to form a colony in the absence of required nutrients is inversely proportional to the quantity of nutrient required. Generally, absence of a suitable carbon source is felt first, amino acids required as growth factors next, and lastly the absence of added vitamins—some of which may be stored in quantities sufficient for many generations. It is highly improbable that suitable carbon sources for any of the fungi with which we were concerned were lacking. As a source of amino acids, gelatin hydrolysate is inferior, since it is poor in methionine and in the aromatic amino acids. Nevertheless, we have maintained methionine-requiring Phycomycetes on gelatin hydrolysate media for over a year of semi-monthly transfers. The feasibility of isolating forms requiring growth factors not present in this medium was demonstrated by the isolation of sterol-requiring *Labyrinthulas* from two of the sea water samples used in this study (4 and 7, Table II). Syntrophism may occur (though no obvious examples were seen) on these plates.

An experimental approach to this problem was made by plating a sample of sea water from *Ulva* colonies on pilings at City Point, New Haven, in triplicate on the isolation medium, on the isolation medium without liver extract, on the isolation medium without liver extract, vitamins, or glucose, and on a medium containing glucose, glutamate, and thiamine as its only organic constituents. At the same time isolates known to require liver extract and known to require glucose, amino acids, and vitamins were plated on these media. Of the media used, only the glucose-glutamate-thiamine agar gave significantly lower sea water counts. This medium also failed to support typical colony formation by isolates known to require growth factors. The isolates requiring liver extract failed to form typical colonies on the medium from which liver, glucose, and vitamins were omitted also, though the omission of liver extract alone did not affect their growth under the conditions used.

It is, on the other hand, quite possible that fungi exist which did not form recognizable colonies because they were inhibited by the ingredients of the medium. Representative colonies were picked from the isolation plates made at Woods Hole into tubes of semi-solid (0.1% agar) isolation medium for further study. It then developed that, in semi-solid media, each of the organic constituents of the medium was somewhat inhibitory to some of the fungi isolated. The results of the comparative plating of City Point sea water outlined above and of similarly plating isolates known to be inhibited by ingredients of the isolation medium indicated that, for these fungi, the inhibitions are relieved by growth on a solid agar surface. The validity of this conclusion must be restricted to the fungi examined.

In principle, these defects are not unique to our procedure; they require restatement here because this is the first application of plating techniques to the lower marine fungi, indeed to ecologic studies of any aquatic Phycomycetes, and because they bear on the conclusions to be drawn from our results. The results of Table II suggest, first, that the presence of these fungi is correlated with the organic content of the water examined, since the highly polluted Eel Pond is richer than Great Harbor (on the incoming tide). Second, in Great Harbor (and in the Hole), fungi are associated with algae: they may be isolated from water taken from finger bowls in which algae were being kept under a constant drip of previously fungus-free tap water. They may be isolated from water squeezed by hand from masses of attached algae growing on rocks and pilings.

The organic content of polluted waters, such as those of the Eel Pond, could reasonably be expected to support a population of free-living non-filamentous fungal saprophytes, just as of bacterial saprophytes. We have calculated, from cell counts of representative cultures, that the amount of soluble organic material required to produce a single thallus of the common holocarpic or monocentric marine Phycomycetes is of the order of 1 mμ g. But the development of techniques for establishing directly the presence of a free-living fungus population would be very desirable. Two instances of association with organic debris were noted. One colony of a monocentric Phycomycete was found on a plate on a stray grain of pine pollen. One species (an undescribed myxoid form here referred to as isolate "S"), of which 1–22 colonies were found on every plate containing lower fungi, formed colonies which were as often as not centered on a microscopic bit of nondescript organic debris.

In view of the known endo- and epibiotic habit of marine fungi, the apparent association with algae required further investigation. The algae with which the sea water samples of Table II were associated were examined microscopically for the presence of fungal thalli. As might have been expected from the experience of previous investigators (see Petersen, 1905; Sparrow, 1934, 1936; Kobayashi and Ookubo, 1953), recognizable thalli were rare. The one species which Sparrow (1936) found epidemic later in the summer—*Ectrogella perforans* in *Licmophora*—was conspicuously absent. The only form found during these examinations was *Petersenia lobata* (?) on *Polysiphonia urceolata* (*fide* W. R. Taylor) collected at the Red Spindle and allowed to rest for 12 days in a finger bowl under dripping sea water (as suggested by Sparrow). Five days later the infection had disappeared.

TABLE III

Fungi associated with algal surfaces

	No. fragments	No. species fungi/fragment
1. Algae from rocks at Red Spindle (Grassy Island)		
Ectocarpus	1	2
Antithamnion	8	1–3
Polysiphonia	4	1–3
Ceramium	3	1–3
2. Algae from rocks of Pine Island		
Ectocarpus	4	1–2
Elachistea	5	1
Punctaria	2	2
Chorda filum	1	3
Callithamnion	18	1–3
Antithamnion	4	2
	1	0
Polysiphonia	3	0
Bryopsis	10	1–2
	4	0

The results of plating small pieces of algae showed, in marked contrast, that the algal surface not contaminated by lower fungi was rare. Bits of algae, usually strands approximately one cm. long, were cut from the collected plants, drawn gently over the edge of a petri dish to remove water and placed on the surface of the usual antibiotic-isolation medium agar. No fungal thalli (possibly because of their small size) could be recognized at 100 × on the algal fragments at the time of plating. Neither fungal thalli nor rhizoids were seen within the cells of these algae at any time, though the specimens were examined at 430 × at the end of the incubation period. The association of lower fungi with fragments of algae collected at the Red Spindle (Grassy Island) in Great Harbor and at Pine Island (off Nonamesset Island, in the Hole) is shown in Table III. Molds and yeasts were observed fairly frequently; specimens in this series which were obscured by such forms have been omitted from the tabulation.

Are these fungi on the algal surface or in the surface film of water surrounding the algal fragment? If the surface film of water is, at a guess, about 0.01 ml., one would expect to find of the order of 1–10 viable units of lower fungi in it. The results of Table III are not inconsistent with this estimate, but are unfortunately not quantitative, because discrete colonies are rarely formed around algal fragments. But we are inclined to consider, for two reasons, that the fungi found in association with algal fragments resemble many of the marine bacteria in being present, as thalli, on the surface of the alga. First, we have attempted (in a limited number of trials) to wash the fungi away with sterile sea water. The results, even with such algae as *Bryopsis plumosa* and *Polysiphonia urceolata* which were free of the forest of hairs and epiphytes found on most marine algae, were poor. No more fragments were free of fungi after washing than before, and essentially the same type of fungi was present. Secondly, although both holocarpic and eucarpic Phycomycetes were among the forms associated with algal surfaces, the predominant form was, as in sea water samples, the myxoid surface-loving "S." Every algal fragment listed in Table III as having associated fungi had "S" associated with it. The association of "S" with bits of organic debris has already been noted. "S" was also found to be associated with two of six copepods plated as part of a rather unproductive plankton haul from Great Harbor. Another surface-loving form, *Labyrinthula* sp., occurred less frequently on *Polysiphonia, Ceramium,* and *Ectocarpus.*

Conclusions and Summary

Our data therefore suggest that the lower marine fungi occupy essentially the same ecologic niche as the marine saprophytic bacteria. These fungi can be found in suitably polluted sea water in numbers of the order of 1–500,000 viable units/liter but less than 5000/liter in more open waters of Woods Hole. The fungus count increases, as has long been noted for bacteria (Gazert, 1906), in the presence of macroscopic algae. They also resemble marine bacteria in their association with surfaces. As a group, these fungi differ from marine bacteria in being strongly aerobic. One may justifiably wonder as to the basis of their success in competition with bacteria in this niche. Studies, now in progress, of the individual species of fungi concerned may clarify this question.

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THE EFFECT AND AFTER–EFFECT OF VARIED EXPOSURE TO LIGHT ON CHICKEN DEVELOPMENT

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The basic factor necessary for initiating development of the gonads of birds, according to Rowan (1938), appears to be length of day. Intensity above a certain low threshold, he says, appears to be of no significance. Frequency of light stimulation plays an important role according to Benoit (1936) and, more recently, Staffe (1951), Kirkpatrick and Leopold (1952), and Farner *et al.* (1953a). On the other hand, with uninterrupted lighting early growth was delayed and reproductive performance adversely affected, as shown by studies of Lamoreux (1943), Callenbach *et al.* (1944), and Ringrose and Potter (1953).

Since most of the studies cited above involved wild birds, it remains to be seen whether their results are directly applicable to the domestic fowl; this is particularly true for strains of chickens that have been bred for egg production. Previous work on the effect of intermittent lighting on laying hens by Wilson and Abplanalp (1956) has indicated that egg production can be maintained with very small amounts of light energy, provided it is given intermittently (less than six one-minute intervals in 24 hours). These results tend to support earlier findings by Staffe (1951), who demonstrated that short light flashes from 1500-watt lamps were effective in stimulating laying hens to increase winter egg production.

The present study was conducted in order to gain further information about the effects of intermittent lighting upon the development of chickens. It was, however, restricted to an investigation of early growth and the onset of sexual maturity in pullets.

Material and Methods

Two experiments were conducted with Single Comb White Leghorn stock of the University of California at Davis. In the first, pedigreed chicks were hatched on December 4, 1953. The purpose of the test was to study the effect of supplementary light on the development of chickens. Each hen's chicks were distributed as equally as possible among six experimental groups. Each lot of chicks was then brooded and reared in 15 × 15-foot pens of a house with open fronts. Natural light was given to all six pens, but three received supplementary artificial light from a continuously burning 100-watt incandescent bulb. Three brooding methods were used with the two light treatments as follows:

Group	Brooder type	Light regime
1	4 infrared lamps	Natural light only
2	Electric	Natural light only
3	Gas	Natural light only
4	4 infrared lamps	Natural light + 100 W continuous
5	Electric	Natural light + 100 W continuous
6	Gas	Natural light + 100 W continuous

The gas brooders provided a somewhat higher room temperature than either of the other types.

Brooding was discontinued when the chicks were 6 weeks old. A week later, at 7 weeks of age, the chicks were scored for feathering. Four grades were used in assessing completeness of feathering, ranging from 1 for poorest feathering to 4 for best performance. Both sexes were scored.

At the same time a few males from large families were killed in order to determine comb and testis weights. Body weight was measured first at 7 weeks of age and from then on at 4-week intervals. Age at first egg and the average weight of the first three eggs were determined whenever possible.

The second experiment was to determine effects of intensity and frequency of lighting upon growth and sexual development of pullets. Pullet chicks were hatched on July 1, 1955, and all brooded alike up to 5 weeks of age. The following brooding and lighting regime was used:

0–1 weeks of age: 10-watt bulb; continuous light
1–3 weeks of age: 40-watt bulb; continuous light
3–5 weeks of age: 10-watt bulb; continuous light

Shielded incandescent light bulbs were the only source of heat.

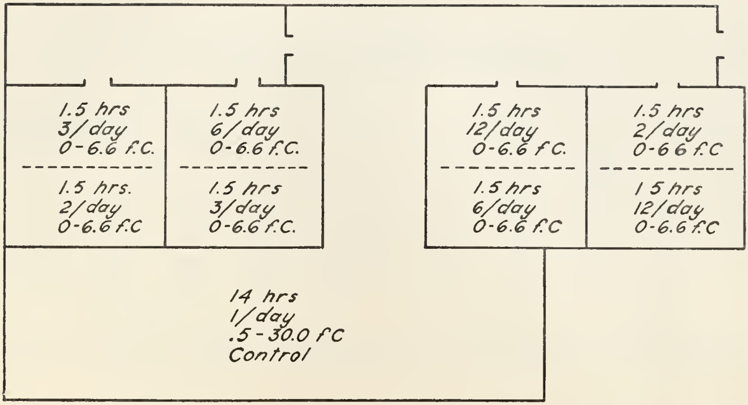
At 5 weeks of age, and in some cases again at 90 days of age, the experimental lots of birds were subjected to changes in lighting regime, according to the following plan:

Group	Light intensity (foot-candles)		Total hrs. light in 24 hours	Number of light periods in 24 hours after 90 days of age
	35–90 days	After 90 days		
A	0.5–30.0	0.5–30.0	14.0	1
B	0.0–0.4	0.5–30.0	14.0	1
C	0.0–0.4	0.0–0.4	1.5	12; 6; 3; 2.
D	0.5–30.0	0.0–0.4	1.5	12; 6; 3; 2.
E	0.5–30.0	0.4–6.6	1.5	12; 6; 3; 2.

As indicated in the table above, groups C, D, and E were each divided into four subgroups at 90 days of age, and the latter were subjected to light periods of varying frequency, while the total duration of lighting was held constant. The 12 light periods per 24 hours consisted of 7.5 minutes each, followed by 52.5 minutes of darkness. Similar regularly spaced periods of light were used where six and three stimuli were given. In the case of two light periods, however, 45 minutes of light was alternately followed by 7 hours, 15 minutes and by 15 hours, 15 minutes of darkness. Group A served as control and was held under "cool white" fluorescent lighting with 14 hours of light per day. Light intensities varied between 0.5 and 30.0 foot-candles according to the location of individual laying cages.

Four windowless climatic chambers were used for this experiment; they have been described in detail by Wilson and Abplanalp (1956). Each chamber was subdivided into two sections with a partition of black sisalkraft, in order to allow replication of treatments. Thus, each lighting regime was given in two different chambers. A diagrammatic outline of this arrangement is given in Figure 1.

Temperatures were held constantly at approximately 80° F. The pullets were


1.5 hrs
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2/day
0-6 6 f.C
1 5 hrs
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0-6.6 f.C
14 hrs
1/day
.5 - 30.0 f.C
Control

FIGURE 1. Floor plan of experimental rooms showing location of replications. Total light per day, number of light periods per 24 hours, and intensity in foot-candles are given in order for each subgroup.

placed in individual 10-inch cages when they were 5 weeks old. Two double rows of cages were arranged in step fashion, which meant that the birds in the upper rows were somewhat closer to the light source than those in the lower cages. The top rows were 18 inches from the light source, while the bottom ones were 3 feet distant. Light intensities varied between 0.0 and 0.4 foot-candles for the lower tiers and between 0.4 and 6.6 foot-candles for the upper ones.

Experimental lighting was held at suboptimal intensities in order to bring out more clearly the possible effects of frequency of light periods.

The following traits were observed and analyzed: 1) Body weights at 12, 20, and 28 weeks of age; 2) age at first egg for individual pullets; and 3) average

TABLE I

Effect of supplementary light on growth, feathering and sexual development of December-hatched chicks. Number of individuals in parenthesis

C–22	Body weight, females gms.				Testes wt. at 7 weeks (gms.)	Feather score	Aver. age at 1st egg (days)	Aver. wt. 1st 3 eggs (gms.)
	7 wk.	12 wk.	16 wk.	20 wk.				
No artificial lights	(189) 436	(189) 1023	(189) 1322	(189) 1605	(36) .240	(553) 3.47	(189) 157.7	41.0
100-watt lights cont.	(190) 452	(190) 1006	(190) 1303	(190) 1519	(32) .284	(545) 3.21	(190) 163.8	43.6
Difference	−16*	17	19	86**	−.044**	.26**	−6.1*	−2.6**

* = $P < .05$.
** = $P < .01$.

TABLE II

Relation of light intensity at different stages of growth and age at first egg. Group A and B received 14 hours of light after 90 days of age, other groups 1½ hours

Group	No.	Light treatment foot-candles		Body weight (gms.)			Median age at 1st egg (days)	Aver. wt. 1st 3 eggs (gms.)	At 248 days % maturity
		Between 35–90 days	After 90 days	12 wk.	20 wk.	28 wk.			
A	10	0.5–30.	0.5–30.	1031.	1514.	1744.	153.	39.7	100
B	10	0–0.4	0.5–30.	990.	1505.	1779.	161.	42.2	100
C	40	0–0.4	0–0.4	1017.	1391.	1707.	191.	48.9	85
D	40	0.5–30.	0–0.4	1013.	1466.	1838.	179.	47.5	72
E	40	0.5–30.	0.4–6.6	958.	1426.	1705.	169.	45.1	95

weight of first three eggs laid (whenever possible). Median age at first egg was used as a measure of sexual maturity of entire groups of pullets.

RESULTS

The results of the first test are shown in Table I. Continuous light added to natural illumination apparently favors early growth of chicks up to at least 7 weeks of age. Later on, and most conspicuously at 20 weeks of age, the effects of continuous light supplement are just the opposite. Significantly higher body weights were found at 20 weeks for the birds brooded under natural light only.

Comb weight of 7-week-old males shows little or no effect as the result of different light treatments, but testis weights responded in the same way as body weight. Added continuous lighting resulted in significantly heavier testes at 7 weeks of age.

Continuous light tends to retard feathering and sexual maturity of pullets (but not of males). The 6-day differences in maturity between supplemented and control groups was highly significant. This delay in age at first egg was associated with a somewhat higher weight of first eggs.

The results of the second experiment are given in Tables II and III. In Table II the data are arranged according to total amount of daily lighting and light intensities. Table III, on the other hand, shows the effects of increasing frequencies of light periods with a given amount of light applied after 90 days of age.

TABLE III

Frequency of light intervals/24 hrs. in relation to growth and age at first egg

Light foot-candles	Light periods/24 hrs.		Total light/24 hrs.	No.	Aver. body weight (gms.)			Median age at 1st egg (days)	Aver. wt. 1st 3 eggs (gms.)
	Number	Length			12 wk.	20 wk.	28 wk.		
0–6.6	12	7½ min.	90 min.	30	991.	1437.	1751.	168.0	46.5
0–6.6	6	15 min.	90 min.	30	980.	1410.	1736.	174.0	46.1
0–6.6	3	30 min.	90 min.	30	1011.	1481.	1826.	190.5	48.0
0–6.6	2	45 min.	90 min.	30	1002.	1381.	1750.	189.0	47.6
0.5–30.	1	14 hrs.	14 hrs.	20	1010.	1510.	1759.	160.0	41.2

The observed body weights of pullets do not show any clear-cut effects of either light intensity, amount of light, or lighting frequency.

Sexual maturity as measured by median age at first egg, however, was strongly affected by differences in lighting, both before and after 90 days of age. The control treatment (group A) with highest light intensity during early and late periods of development matured earliest. Group B, with low light intensity to 90 days of age but high intensity thereafter, matured 8 days later. Similarly, groups E, D, and C show consistently adverse effects of reduced light intensities upon age at first egg.

The influence of light intensity on maturity is apparently operative over a considerable period of early development and is not merely restricted to a period very close to the onset of egg production. This can best be shown by regrouping the results of median age at sexual maturity.

Light before 90 days of age	Light after 90 days of age		
	Dim	Bright	Difference
Dim	191	161	30
Bright	179	153	26
Difference	12	8	—

Here it may be noted that bright light administered before pullets were 90 days old advanced sexual maturity by only 12 and 8 days, while intensive lighting after 90 days of age produced effects of 30 and 26 days. This clearly indicates that the influence of light intensity upon sexual maturity becomes more pronounced the closer toward onset of lay it can operate.

Table III shows the effects of more frequent light periods on age at first egg. In each case the birds received a total of 90 minutes of dim light in 24 hours. The results are clear-cut and show that light is more effective in stimulating sexual development the more frequently it is applied. Thus, 12 short periods of dim light permitted pullets to mature almost as early as one 14-hour period of intensive light. The same amount of dim light given in only two doses, on the other hand, resulted in extremely late maturity. These findings are shown more clearly in Figure 2. The data represent medians for the replicate lots. Each lot contained subgroups which differed in light intensity. See regime for groups C, D and E.

Discussion

The first experiment confirmed that growth of the domestic fowl can be influenced by light. All-night lights may have aided early growth in this experiment by providing the chicks more opportunity to feed. Frequent feedings may be particularly helpful when the crop capacity of chicks is still poorly developed.

The adverse effects of continuous lighting after 7 weeks of age, as found in this study, are in agreement with findings by Tomhave (1954).

The results of the second test with respect to growth after 12 weeks of age are not conclusive. They fail to substantiate reports by Clegg and Sanford (1951) and by Barott and Pringle (1951), who found that intermittent lighting has a beneficial effect on early growth of chickens prior to 12 weeks.

In this study it has been found that continuous light has adverse effects on feathering, becoming more serious as the birds become older. Similar effects of continuous light on turkeys have been reported by Mueller *et al.*, 1951.

The role of continuous light in the sexual development of chicks appears to be a complex one. While the 7-week-old cockerels showed increased testes weight under continuous lighting, the pullets appeared to be delayed in their development. Unfortunately, there were no testes measurements available for cockerels near maturity; hence, it is difficult to assess the possible importance of refractory behavior of pullets. Evidence of retarded growth at 20 weeks of age indicates, however, that both cockerels and pullets may have been delayed sexually near the point of maturity.

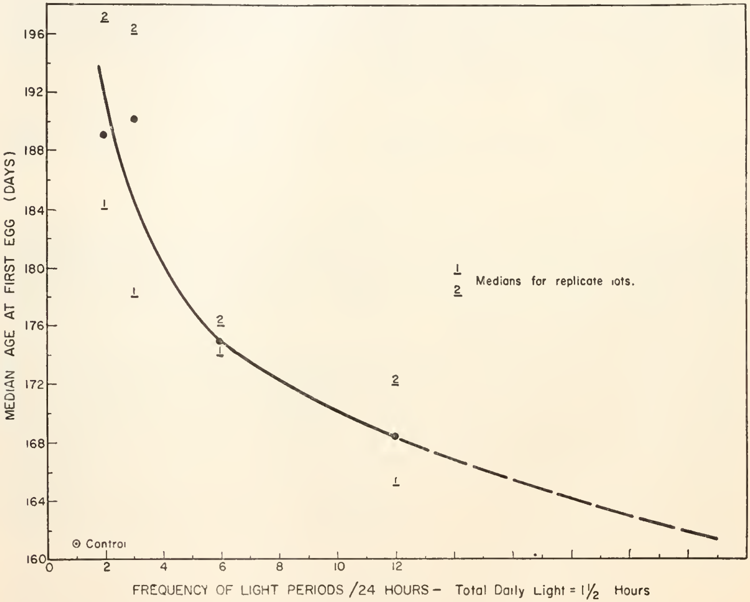

FIGURE 2. The relation of frequency of light periods and age at first egg.

From the results of the second experiment, as well as from an earlier report by Wilson and Abplanalp (1956), it is clear that the total amount of light is not the sole determinant in stimulating sexual development of pullets. Aside from the adverse effects of continuous lighting (which may possibly be due to a nervous fatigue of the animal), it has also been demonstrated here that light intensity and the distribution and frequency of light stimuli are important variables that must be considered in the problem of light stimulation.

The present results show that under limiting conditions of low light intensities and short periods of light exposure, the rate of sexual development increases with longer total daily exposure to light, greater light intensity, and more frequent stimulation. These relationships may not hold when either light intensity or total duration of light exposure is increased beyond certain thresholds. Nevertheless, they permit certain conclusions of practical value. Thus, where natural light is to be supplemented, it would seem reasonable to use frequent but short intervals of artificial lighting in place of continuous lighting or of light periods adjoining the natural day.

Several attempts have been made recently to rationalize the response of birds to light. Wolfson (1953) interprets his data as supporting the hypothesis that the total daily dose of light determines the response. He postulates, however, that the proportion of light exposure to darkness in a given cycle is the critical factor in determining the response rather than the daily dose of light.

Kirkpatrick and Leopold (1952), in agreement with Jenner and Engels (1952), maintain that the dark period *per se* is a major controlling factor in the response of birds to light. Such an interpretation appears primarily different in terminology and emphasis but seems to add little to an understanding of the problem. It has been criticized by Hammond (1953) and by Farner *et al.* (1953b).

In the light of this study, the theory advanced by Farner *et al.* (1953b) seems the simplest and most suitable for explaining the action of light in reproduction of birds. In brief, it postulates that there exists a light-sensitive gonadotropic mechanism capable of activation almost immediately upon onset of lighting; it remains active throughout the light period and even for some time following termination of the latter. This theory has helped to explain the effectiveness of extremely short photoperiods (a total of 6 minutes in 24 hours) in maintaining egg production of chickens (Wilson and Abplanalp, 1956). We believe this theory suitable to explain the current findings which show the increasing effectiveness of a given amount of light when given in numerous small doses.

The formulation of general theories on the basis of published evidence is seriously hampered by non-uniformity in experimental material, procedures, and terminology. On the basis of Farner's theory and present findings, one may attempt to interpret light response by means of three independent main effects and their interactions, namely:

1. Total daily amount of light exposure
2. Light intensity
3. Frequency of light intervals.

Additional assumptions are needed in order to explain refractoriness and perhaps seasonal changes in reproduction of birds, but the present experiments do not permit any new interpretation of their role.

SUMMARY

The present experiments were designed to determine the effects of:

a. Total daily amount of light
b. Intensity of light
c. Frequency of light intervals

on the growth and development of Leghorn chickens.

a. *Total daily amount of light exposures:* The first test dealt with the effect of supplementing natural light with continuous light. Continuous light improved body weight of all birds and testes size of males at 7 weeks, but impaired feather development. Continuous lighting delayed sexual maturity of pullets, and growth in both sexes was retarded until they reached 20 weeks of age.

In the second experiment under suboptimal light intensities, light exposure has no effect on body size after 12 weeks of age. Rate of sexual development was increased by larger daily exposures to light. These effects were more pronounced when treatments were applied to pullets over 90 days old than during earlier stages of development.

b. *Intensity of light:* Three light intensities were applied to growing birds: 0.0–0.4 foot-candles, 0.4–6.6 foot-candles, and 0.5–30.0 foot-candles. Growth was not affected by lower light intensities, but sexual maturity was delayed.

c. *Frequency of light intervals:* Body weight was unaffected by lighting frequency. Sexual maturity, however, was significantly advanced when suboptimal light exposure and light intensities were applied in frequent but small doses. Thus, it was found that 12 periods of 7.5 minutes of dim light per day produced a rate of sexual development almost equal to the rate with 14 hours per day of normal lighting. These results are taken as further proof that the after-effects of light on the reproductive mechanisms of chickens are considerable

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